Host cell death modulation by *Chlamydia trachomatis*

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Zusammenfassung

Chlamydien sind gram-negative humanpathogene Bakterien, die eine Vielfalt an Krankheiten im Menschen verursachen. Dazu gehören die durch Infektion verursachte Blindheit wie auch zahlreiche sexuell übertragbare Krankheiten. Chlamydien sind obligat intrazelluläre Bakterien; sie entwickeln sich innerhalb einer membranumhüllten Vakuole innerhalb der Zelle, der sogenannten Inklusion. Ihnen ist ein einzigartiger biphasischer Entwicklungszyklus zueigen, während dessen sie teilweise von der Wirtszelle abhängig sind.

Das Immunsystem des Wirts hat die Fähigkeit, Zellen, die Bakterien tragen, zu erkennen und zu eliminieren. Infizierte Zellen exprimieren vermehrt MHC Klasse I oder II Moleküle auf der Oberfläche, die durch CD+ T Zellen erkannt werden. Daraufhin wird die Zelle durch Apoptose zerstört. Apoptose ist eine Form des programmierten Zelltods, die eine Kaskade von biochemischen Ereignissen beinhaltet, die zu einer systematischen Auflösung des Zellapparates und dessen Entfernung aus dem Organismus führt, ohne eine Entzündung auszulösen. Chlamydien haben verschiedene Strategien entwickelt, um vom Immunsystem unerkannt zu bleiben oder die Wirtszelle vor der Zerstörung zu bewahren. Diese hervorragende Eigenschaft der Chlamydien, Wirtszellen effektiv vor der Apoptose zu schützen, hilft ihnen, lange genug im Wirt zu überleben und zu replizieren, um ihren Entwicklungszyklus erfolgreich zu beenden.

Gegenstand der vorliegenden Arbeit war es, die Mechanismen aufzuklären, durch die Chlamydien die Wirtszellapoptose regulieren. Dabei wurde *Chlamydia trachomatis* als ein Modellorganismus gewählt.

Es konnte beobachtet warden, dass Chlamydien durch die Modulation spezifischer Wirtszellproteine verschiedene Wege der Apoptose verhindern können. Mcl-1 und cIAP-2 erwiesen sich als bedeutende Faktoren, die durch die Infektion hochreguliert und absolut notwendig für die Inhibierung der Apoptose durch Chlamydien waren. Hochregulation der Mcl-1 Expression führte zu einem Block im apoptotischen Weg oberhalb der Mitochondrien. cIAP-2 zusammen mit anderen Inhibitor of Apoptosis Proteins (IAP) verhinderten die Aktivierung von Caspase-3, denfinalen Schritt in der apoptotischen Kaskade. Weiterhin wurde beobachtet, dass die Aktivierung des MAPKinase-Signalweges durch die Infektion wichtig war für die Hochregulierung von Mcl-1 und cIAP-2.

Eine interessante Beobachtung war, dass cIAP-1, cIAP-2 und XIAP miteinander interagieren und in hochmolekularen heteromerischen Komplexen in der Zelle existieren. Abwesenheit von XIAP verhinderte die Bildung dieses Komplexes. Es wurde auch beobachtet, dass der IAP Antagonist Smac wie auch Caspase-3 während der Apoptose zum IAP-Komplex rekrutiert werden. Daraus konnte gefolgert werden, dass der IAP-Komplex eine funktionale Rolle bei der Regulierung der Caspase-Aktivierung spielt.

Ein Hochdurchsatz-Screen wurde durchgeführt, um andere Wirtszellfaktoren, die für die Apoptoseinhibierung durch die Chlamydien verantwortlich sind, zu identifizieren. Neben Mcl-1 waren die identifizierten Faktoren hauptsächlich Mitglieder des MAP Kinase-Signalweges. Dabei wurde deren Rolle für die Apoptoseresistenz bestätigt. Eine weiterführende Analyse der im Screen ermittelten Faktoren identifizierte eine Funktion von HIF-1 alpha bei der Modulation der Expression anti-apoptotischer Faktoren während der Infektion. Es wurde beobachtet, dass HIF-1alpha stabilisiert und zum Nukleus transloziert wird. Es ist bekannt, dass HIF-1alpha HIF-1beta im Nukleus binden kann, um den funktionalen Transkriptionsfaktor HIF zu bilden. Dieser reguliert die Expression verschiedener Überlebensfaktoren, unter anderem Mcl-1. HIF-1 alpha Knockdown inhibierte die Chlamydien-induzierte Hochregulation von Mcl-1 mRNA-Expression.

Die vorliegende Arbeit zeigt, dass die Chlamydien-Infektion die Apoptose an verschiedenen Stellen inhibiert. Die Infektion führt zur Hochregulation von anti-apoptotischen Faktoren, inklusive Mcl-1 und cIAP-2, in Abhängigkeit von der Aktivierung von MAPKinasen. HIF-1alpha wird durch die Infektion stabilisiert, wahrscheinlich durch infektionsbedingte Hypoxie, und bewirkt dietranskriptionelle Hochregulation von Mcl-1 und cIAP-2.

Daraus kann geschlussfolgert werden, dass die chlamydiale Infektion ein interessantes Szenario darstellt, bei dem die Bakterien die Zelle invasieren und diese vor dem Zelltod schützen, indem sie Wirtszellüberlebenssignalwege aktivieren. Es gibt wachsende Evidenz dafür, dass Chlamydien-Infektionen mit verschiedenen Krebsarten im Zusammenhang stehen. Das Verständnis der Modulation von SignalwegenderWirtszelledurch Chlamydien kann bei der Aufklärung der Rolle chlamydialer Infektionen in der Tumorentstehung und –progression hilfreich sein.

Summary

Chlamydia are gram-negative pathogens causing widespread diseases in humans including infectious blindness as well as sexually transmitted diseases. These are obligatory intracellular and live inside a membrane bound vacuole, termed inclusion, in the cell. *Chlamydia* are dependent on the host cell to meet their nutritional demands during their biphasic life cycle.

The host cell immune system has the ability to recognize and eliminate cells that are carrying most bacterial infections. CD+ T cells can identify infected cells by the surface expression of MHC Class I or II molecules and subsequently destroy the cell by the process of apoptosis. Apoptosis is a form of programmed cell death involving a chain of biochemical events which lead to systematic dismantling of the cellular apparatus and its removal in an ordered way. However, *Chlamydia* have evolved various strategies whereby they not only prevent the detection of the host cell by the immune system, but also protect it from destruction by the immune system in case of recognition. This remarkable ability of *Chlamydia* to robustly protect host cells from apoptosis helps it to survive long enough in the host cell to complete the life cycle.

The present study was aimed at determining the mechanisms by which *Chlamydia* interfere with the apoptosis induction in host cells, using *Chlamydia trachomatis* as a model organism.

It was seen that chlamydial infection blocked the apoptotic pathway at multiple levels by modulation of specific host cell proteins. Mcl-1 and cIAP-2 were two most prominent factors that were up-regulated during the infection, and absolutely required for apoptosis inhibition. Increased expression of Mcl-1 led to a block in the apoptotic pathway upstream of the mitochondria. cIAP-2, together with other inhibitor of apoptosis proteins (IAPs), blocked the activation of caspase-3 at the final step of the apoptosis cascade. Further, it was observed that the activation of the MAPK pathways during infection was needed for the up-regulation of Mcl-1 and cIAP-2.

An interesting observation was that cIAP-1, cIAP-2 and XIAP interact with each other and exist in a high molecular weight heteromeric complex in cells. Absence of XIAP was seen to disrupt the complex. It was also seen that the IAP antagonist Smac as well as caspase-3

get recruited to the IAP complex during apoptosis induction, suggesting that the IAP complex has a functional role in regulating caspase activation during apoptosis.

A high throughput RNAi screen was performed to identify other host factors required for the apoptosis resistance during the infection. Besides Mcl-1, the targets from the screen prominently included members of the MAPK pathways, confirming their role in the apoptosis resistance. Pathway analysis of the targets identified the role of HIF-1 alpha in modulating the expression of the anti-apoptotic factors during infection. It was observed that during infection, HIF-1 alpha gets stabilized and translocates to the nucleus. It is known that HIF-1 alpha can bind to HIF-1beta in the nucleus to form the functional transcription factor HIF, which can regulate the expression of survival factors like Mcl-1. This was seen to be the case, because knock down of HIF-1 alpha abrogated the infection induced up-regulation of Mcl-1 at the mRNA levels.

From the present work it can be concluded that chlamydial infection blocks the apoptotic pathway at various levels. The infection leads to the up-regulation of prominent anti-apoptotic factors including Mcl-1 and cIAP-2 in a MAPK dependent fashion. HIF-1 alpha gets stabilized in the infected cells, probably due to infection induced hypoxic conditions and subsequently results in the transcriptional up-regulation of Mcl-1 and cIAP-2.

Thus the chlamydial infection presents an intriguing scenario where bacteria invade a cell and then protect it by activating the host survival pathways. Moreover, there is growing evidence relating chlamydial infection with certain cancers. The understanding of the chlamydial modulation of cell signalling will be helpful in unravelling the mechanisms of the establishment of chlamydial infection *in vivo* and its role in tumor progression.

Abbreviations

AIF	Apoptosis Inducing Factor
Apaf1	Apoptosis activating factor-1
APS	Ammonium Per Sulphate
BAD	Bcl-2-associated death promoter
Bcl-2	B cell lymphoma 2
BH	Bcl-2 Homology
BID	Bcl-2 interacting domain
BIM	Bcl-2-interacting mediator of cell death
BIR	Baculovirus Immuno Repeat
CAD	Caspase Activated DNAase
CARD	Caspase Recruitment domain
c-FLIPs	FLICE/caspase-8 like Inhibitory Protein
CHX	Cyclohexamide
CTL	Cytotoxic T Cell
Ctr	Chlamydia trachomatis
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo Nucleic Acid
EB	Elementary Bodies
ER	Endoplasmic Reticulum
FADD	Fas associated death domain
GFP	Green fluorescence protein
HEPES	N-(2-hydroxyethyl) piperazine-N`-(2-ethanesulfonic acid)´
HIF-1	Hypoxia Inducible Factor
HIF-1a	Hypoxia Inducible Factor-1a
IAP	Inhibitor of apoptosis protein
IFU	Inclusion Forming Units
Kb	kilo base pairs
MAPK	Mitogen Activated Protein Kinases

Mcl-1	Myeloid Cell Leukemia-1
MEKK1	MAPK/ERK kinase kinases
MEM	Minimal Essential Medium
MHC	Major Histocompatibility Complex
MM	Maintenance Medium
MOMP	Mitochondrial Outer Membrane Permeabilisation
MOI	Multiplicity of Infection
MOMP	Major Outer Membrane Protein
NFkB	Nuclear factor kB
PARP-1	poly(ADP-ribose)polymerase 1
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
RB	Reticulate Bodies
RNA	Ribo nucleic acid
rRNA	ribosomal ribo nucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-poly acrylamide gel electrophoresis
SH	Src-homologue
Smac/DIABLO	second mitochondrial activator of caspases/direct IAP binding protein with low pl.
STS	Staurosporine
tBid	Truncated Bid
TBS	TRIS-buffered saline
TEMED	N,N,N'N'-tetramethylethylenediamide
TNF-a	Tumour Necrosis Factor-a
TNFR1	TNF receptor-1
TRAIL	TNF-Related Apoptosis-Inducing Ligand
KDa	Kilo Dalton

VDAC	Voltage Dependent Anion Channel

1 Introduction

Interactions between bacteria and host cells are of diverse kinds. In an early stage of life on earth, a bacterial invasion into an archaeon cell led to an endosymbiotic relationship between the two [1]. The parasitic bacteria contributed to the metabolism of the host cell through respiration while taking up nutrition from the cell. Eventually this endosymbiosis resulted in the evolution of an organelle in the cell - the mitochondria, which became responsible for the respiration in the cell. However, it appears that along with the function of respiration, another role has been passed over to the present day eukaryotic cell as a legacy from the initial incorporation of the aerobic bacteria into the ancestral eukaryote – that of apoptosis [2]. The present day eukaryotic apoptotic machinery seems to be formed of several apoptotic factors originating as a consequence of the mitochondrial endosymbiosis and newly emerging eukaryotic domains that act as adaptor molecules [3]. Mitochondria play a central role in the life and death decisions of a cell. It is well accepted that once the mitochondrial outer membrane gets permeabilized, the apoptotic factors released into the cytosol irreversibly commit the cell to apoptosis.

A billion years hence, in a contrasting mode of interaction between another bacterium, *Chlamydia*, and eukaryotic cells, the bacterium blocks the apoptotic machinery of the host cell. In a reversal of roles, here is a pathogen that strongly protects host cells against destruction by their own organism [4].

1.1 Chlamydia

Chlamydia refers to a genus of obligate intracellular bacteria having a unique biphasic life cycle. Initially classified as viruses, these were later shown to be gram-negative bacteria on the basis of their cell wall architecture and composition apart from their growth by binary division [5]. Many chlamydial species are pathogenic, responsible for a diseases such as blinding trachoma and respiratory tract diseases in humans.

1.1.1 Chlamydial taxonomy

Based on the taxonomic analysis of the 16S and 23S rRNA genes the order Chlamydiales is categorized into distinct groups at the family level including *Chlamydiaceae*, *Parachlamydiaceae* and *Simkaniaceae* [6]. The family *Chlamydiaceae* consists of two genera, *Chlamydia* and *Chlamydophila*. The *Chlamydia* genus includes *Chlamydia*

trachomatis, Chlamydia muridarum and *Chlamydia suis*, while *Chlamydophila* contain *Chlamydophila pecorum, Chlamydophila pneumoniae*, *Chlamydophila psittaci, Chlamydophila abortus*, *Chlamydophila caviae* and *Chlamydophila felis*.

The genomes of several of the chlamydial species have been determined. For example, *Chlamydia trachomatis* serovar D genome contains 1,042,519 nucleotides and an estimated 894 protein-coding genes. It is also reported to contain an extrachromosomal plasmid of 7493 nucleotides [7].

1.1.2 Chlamydial diseases, medical significance

Chlamydia have been implicated in various diseases affecting not only human beings but also several animals.

Chlamydia trachomatis is responsible for the most common sexually transmitted diseases, as well as potentially blinding trachoma, throughout the world. *C. trachomatis* can be classified into at least 15 serologically distinguished serovars (serovariants) based on immunogenic epitope analysis of the major outer membrane protein (MOMP) with polyclonal and monoclonal antibodies. These serovars can be divided into two biovars (biovariants): trachoma and Lymphogranuloma venereum (LGV). The trachoma serovars (A-C) invade mucosal epithelia in the ocular tissue which can lead to endemic blinding trachoma. The serovars D-K infect the urogenital tract causing sexually transmitted diseases. The LGV serovars L1, L2 and L3 invade lymph nodes causing the sexually transmitted systemic syndrome LGV (lymphogranuloma venereum) [8,9,10].

Chlamydophila pneumoniae causes human respiratory tract diseases, with implications in around 6-25 % of community acquired pneumonia. Besides pneumonia, *C. pneumoniae* is also responsible for infections including pharyngitis, sinusitis, otitis, acute bronchitis, persistent cough, chronic obstructive pulmonary disease and Flu – like syndrome (Grayston et al., 1986; Grayston, 1992). Less common infections include extra-respiratory acute infections which might lead to cardiovascular diseases like acute myocarditis, pericarditis and endocarditis or neurological disorders like encephalitis, meningitis, Alzheimer's disease or Guillain-Barre syndrome [11,12]. In addition, seroepidemiology studies have shown an association with coronary artery disease [13,14].

Chlamydophila psittaci and *Chlamydophila pecorum* were implied in several animal diseases. *C. psittaci* infections are widespread in nature, with almost all avian species and most domestic animals being host to the pathogen. *C. psittaci* is responsible for a number

of diseases including enteritis, urogenital infections and pneumonia. *C. psittaci* can also lead to psittacosis in humans [15]. *C. pecorum* infects ruminants and swine causing diseases such as polyarthritis ("stiff-lamb" disease), conjunctivitis, pneumonia, metritis, encephalomyelitis and inapparent enteric infections [16].

Recently, novel species of *Chlamydia* have been discovered including *Parachlamydia acanthamoebae* and *Simkania negevensis*, and implicated as human respiratory pathogens. Studies have also shown that free-living amoeba could support chlamydial multiplication and thus act as reservoir or vector of chlamydial infections [17].

1.1.3 Life cycle of Chlamydia

Chlamydiae have a unique biphasic life cycle, consisting of the infectious Elementary Bodies (EBs) and the metabolically active Reticulate Bodies (RBs). Before infecting a cell, *Chlamydia* exists in the form of the metabolically inactive EBs that are able to survive in the extra-cellular environment. The EBs are approximately 0.2-0.3 μM in diameter and display a condensed nucleoid. After infecting a cell, the EBs differentiate into the RBs, which are around 1 μM in diameter and display a dispersed chromatin structure and are metabolically active. The RBs are metabolically active inside the cell, where they grow in size and multiply by binary fission. Subsequently, the RBs differentiate to form EBs, which are then released from the cell to infect new cells, and continue the life cycle [18,19].

Within 8-12 h after entry, the EB within the vacuole differentiates into the intracellular, metabolically active, replicating RB that measures about 1 μM (Moulder, 1985; 1991). Differentiation of a RB from the EB involves an increase in the size, reduction of the disulfide links in the outer membrane, unraveling of DNA in the condensed nucleoid and the appearance of granular ribosomes [20]. The initiation signal for the differentiation of EB into RB is not known. EBs exposed to reducing environment commence biochemical events such as glutamate oxidation that are thought to possibly be critical for differentiation [21]. However, low pH does not trigger the transition process. The RB multiplies by binary fission in the inclusion, the membrane-bound vacuole, producing daughter cells that are approximately similar in size [19]. By 18 h post infection, a proportion of the developed RBs begins to transform back to EBs, while the remainder continues to divide in the growing inclusion [22]. The environmental signals that induce the reorganization from RBs to EBs are undefined. The RBs continue to reorganize into

EBs in the inclusion that may contain up to a thousand progeny. The organisms acquire ATP, synthetic intermediates and essential metabolites needed for survival from the host cell through the inclusion membrane. At the end of a productive cycle of development, the cell ruptures and the released bacteria infect neighboring cells, repeating the events of the cycle [19]. Although Chlamydiae undergo similar developmental cycle, they differ in the time needed to end one cycle.

1.2 Apoptosis

Cell death is a fundamental aspect of multicellular life forms, with crucial roles during development, and tissue homeostasis. To this end, cell death occurs in a regulated manner, consisting of a well defined sequence of events. Programmed Cell Death, PCD, was first defined as the active physiological process of cell death that is dependent on signalling events in the dying cell [23]. Different types of PCDs were later characterized, including apoptosis (Type I PCD) and autophagy (Type II PCD) [24].

Apoptosis is a form of programmed cell death involving a chain of biochemical events characterized by distinct morphological changes in the cell including cell shrinkage, pyknosis, plasma membrane blebbing, and finally karyorrhexis and formation of apoptotic bodies [25]. Morphologically, a cell dying by apoptosis becomes rounded and retracts from the neighbouring cells. Thereafter, there is a period of dynamic plasma membrane blebbing, resulting in the detachment (pinching off) of the blebs in the form of apoptotic bodies. The apoptotic bodies consist of cytoplasma and tightly packed organelles enclosed within an intact plasma membrane, and are cleared from the system through phagocytosis by macrophages, parenchymal cells or neoplastic cells. Markers like the Phosphotidyl Serine (PS) are exposed on the surface of the apoptotic bodies to be recognised and phagocytosed. During this whole process, there is no release of cellular contents into the surrounding tissue. Moreover, the phagocytes release anti-inflammatory factors upon interaction with the apoptotic cells, resulting in effectively no inflammatory reaction [26].

In contrast to apoptosis, uncontrolled cell ceath (called necrosis) entails a rapid loss of membrane integrity and release of cellular components into the extracellular space, which could lead to damage of the neighbouring cells, and elicitation of the immune response [27].

Dysregulation of apoptosis leads to a variety of human pathologies including cancer, autoimmune diseases and neurodegenerative disorders [28,29,30,31].

Defining features of apoptosis include the condensation and fragmentation of the nucleus, an event that is unique to apoptosis. There is also hydrolysis of the DNA into fragments of sizes that are multiples of 200 bp - a characteristic feature that is used frequently to recognize apoptosis [32]. In addition there is also fragmentation of the golgi apparatus, the endoplasmic reticulum and the mitochondrial networks. The mitochondrial fragmentation has a crucial role in the apoptotic process as discussed below.

During the process of apoptosis, more than 400 proteins undergo restrictive hydrolysis. Although a majority of these proteins cleaved could play some role in the apoptotic events, many cleaved proteins have no known functional relevance and appear to be innocent bystanders during apoptosis [33]. The main proteases involved in this extensive hydrolysis are members of the caspase family [34]. Besides caspases, some other proteases have been characterized in the apoptotic process. These include the lysosomal cathepsins, calpains and the granzymes [35].

1.2.1 Caspases

The primary proteases involved in apoptosis are the caspases or Cysteine Dependent Aspartate Specific Proteases [34]. The caspase gene family thus far contains at least 14 mammalian members, of which 11 human enzymes are known [36]. Caspases and their homologues are known to be present in diverse species including nematodes, yeast, and dipterans [37,38].

The caspase family members share similarities in the amino acid sequence, structure and also the substrate specificity among each other [39]. They are synthesized as inactive zymogens in the cells and are activated either by autocatalytic processing initiated by adaptor-protein mediated aggregation or by proteolysis by other active caspases [40]. The proenzymes are around 30 to 50 kD in size and consist of three domains: a small subunit (~10 kD), a larger subunit (~20 kD), and an NH_2-terminal domain [40]. The activation of the caspases starts with the proteolytic processing between the domains, followed by the formation of a heterodimer by association of the large and the small subunit. The NH-2 domain varies highly in sequence and length, and has been shown to be important for the regulation of activation.

Caspases are extremely specific proteases, with an almost absolute requirement for cleavage after an aspartic acid in the substrate. There are some observed exceptions to this rule- some caspases can unusually cleave substrates after a Glutamate [41,42]. The cleavage site is also marked by the presence of at least four amino acids towards the NH_2-terminal of the site. The tetrapeptide recognition motif (designated as P4-P3-P2-P1) differs among caspases and could explain their functional diversity [43]. It has been seen that the P3 position is invariantly Glutamate for all mammalian caspases [40]. Thus the specificity of cleavage for mammalian caspases can be described as X-Glu-X-Asp. However, not all proteins containing the optimal tetrapeptide sequence are cleaved by caspases, implying that tertiary structural elements are also important for substrate recognition. This strict substrate specificity explains why apoptosis does not entail indiscriminate protein digestion, but instead consists of the cleavage of specific proteins in a coordinated fashion. The protease activity of the caspases is highly efficient ($k_{cat}/K_m > 10^6$ M^{-1} s^{-1}) [40].

Since caspases are potent proteases, they exist in the form of inactive zymogens with a very low enzymatic activity in healthy cells. The activation of caspases is tightly regulated within the cell. The cleavage of the proenzyme is seen to occur at caspase consensus sites, implying that the activation Caspases that are activated initially in response to apoptotic stimuli are called the "initiator caspases", which include caspase-2, -8, -9 and -10. These can in turn activate the downstream "effector caspases", which include caspase-3, -6 and -7. An initiator caspase is characterised by an extended N-terminal prodomain (>90 amino acids) which is important for its function while the prodomain of an effector caspase has a short 20-30 residues (Thornberry and Lazebnik, 1998).

They have been shown to get activated by an autocatalytic process after they are brought in close proximity to each other by adaptor proteins [44]. It has been shown that protein-protein interaction motifs on the caspases viz. the Caspase Recruitment Domain (CARD) and the Death Effecter Domain (DED) interact with similar motifs present on the adaptor proteins to bring the caspases together for the autocatalytic activity [45]. Caspase-8 activation occurs following sequestering by the adaptor protein Fas-Associated Death Domain (FADD) into the Death Inducing Signaling Complex, DISC [44]. Caspase-9 similarly gets activated by forming a heteromeric complex, termed apoptosome with the adaptor protein Apoptotic Protease Activating Factor 1 (Apaf-1) [46]. The initiator procaspases remain as monomers in the cell, and dimerize at the DISC or the apoptosome,

which is required for the formation of the active site. Once activated, the initiator caspases can cleave and activate the effector caspases.
The caspase activation is kept under tight control in the cell. The most prominent family of proteins involved in caspase inhibition is the Inhibitor of Apoptosis proteins, IAPs.

1.2.2 The Inhibitor of Apoptosis Proteins

The Inhibitor of Apoptosis Proteins constitute a family of proteins characterized by the presence of one to three Baculoviral IAP repeat (BIR) domains. The *iap* gene was first identified from insect cells infected with baculovirus, where the gene imparted apoptosis resistance in the host cell [47]. The characteristic BIR domain consists of around 70 amino acids containing the sequence CX2CX16HX6C. The BIR domains fold as a three stranded β-sheet surrounded by four α Helices that form a hydrophobic core with a zinc ion at the centre. Some IAPs also have a RING (Really Interesting New Gene) domain at the C terminus. Examples of such IAPs include cIAP-1, cIAP-2 and XIAP. The RING domains can confer E3 ubiquitin ligase activity to the IAPs conferring to the self-regulation of the IAP proteins by degradation by the proteasome [48]. Other structures present in some IAPs are the Caspase Activation and Recruitment Domain (CARD), Phosphate loop and Ubiquitin-conjugating (UBC) domain.
IAP family members are highly conserved homologs in diverse species ranging from viruses, yeast, nematodes, flies to higher vertebrates. In addition IAP like proteins, ILPs have been found even in plants, where they serve similar functions of apoptosis resistance [49]. XIAP, cIAP1 and cIAP2 are the best characterized mammalian IAPs, each containing three BIR domains and one RING domain.
The three BIR domain subtypes, BIR1, BIR2 and BIR3 are responsible for mediating protein-protein interactions. Although they have an overall similarity, the individual BIR domains have specificities for distinct intermediates involved in different apoptotic and signalling pathways. BIR2 and BIR3 have been shown to bind directly and inhibit the proteolytic activity of caspases [50]. BIR2 selectively targets caspase-3 and caspase-7 while BIR3 is specific for caspase-9 [51]. The caspase binding specificity of BIR2 and BIR3 domains in cIAP-1 and cIAP-2 is similar in extent [50]. All known RING containing IAPs have a ubiquitin ligase activity, targeting a variety of substrates involved in apoptosis and signalling to proteasomal degradation. Interestingly, XIAP, cIAP-1 and cIAP-2 can

catalyze their own ubiquitination in a RING dependant manner when subjected to certain apoptotic stimuli [52,53]. This autoubiquitination is an important regulatory step. It has also been seen that SMAC, once released into the cytosol during apoptosis, can similarly promote the polyubiquitination of cIAP-1 and cIAP-2 leading to their degradation [54,55].

IAPs are best known for their function as apoptosis inhibitors. Early reports showed that IAPs could directly bind to caspases and inhibit their enzymatic activity [56,57]. In mammals, caspases-3, -7 and -9 are subject to inhibition by IAPs [58]. Overexpression of any one of IAPs, such as XIAP, cIAP-1 or cIAP-2 protects cells from apoptosis. It has been observed that although Caspase-9 binds to several IAPs, it is mainly inhibited by XIAP. On the other hand, caspase-3 and -7 are inhibited by XIAP and also by cIAP-1, cIAP-2 and NAIP [56,59]. XIAP is thus the most potent among the IAPs, with its BIR2 domain and the linker between the BIR1 and BIR2 specifically binding to the IAP Binding Motif (IBM) of the active site on caspase-3 and -7 [60].

The mechanisms of apoptosis inhibition of cIAP-1 and cIAP-2 are different from those of XIAP- although both of the former have a caspase binding scaffold, there is no significant caspase-inhibitory activity seen in *in vitro* experiments [50]. It appears that a BIR domain must have an IBM interacting groove as well as an inhibitory element to block caspase activity. However the RING domain on IAPs has been shown to impart an E3 ubiquitin Ligase activity. As such, the RING containing IAPs are known to aid the degradation of many proteins including caspase-3 and caspase-7. Interestingly, cIAP-1 and cIAP-2 are also known to undergo autoubiquitination and cross-ubiquitinate in a RING dependant manner during apoptosis [52,61].

The role of IAPs in apoptosis inhibition is exemplified in the LPS treatment of macrophages derived from mice. Wild type macrophage cells have a transcriptional up-regulation of cIAP-2 during LPS treatment and thus resist apoptosis. In contrast, c-IAP2 knock out macrophages undergo rapid Fas-induced apoptosis upon LPS treatment [62]. In a normal healthy cell, the constitutive levels of IAPs are not sufficient to antagonize apoptotic signals. However, when expressed in high levels, the IAPs are able to effectively block apoptosis.

1.2.3 Pathways of Apoptosis

Apoptosis induction and subsequent caspase activation occurs through two main pathways viz: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. A

third route is involved in cytotoxic T cell-mediated apoptosis initiated by perforin/granzyme (Figure.1).

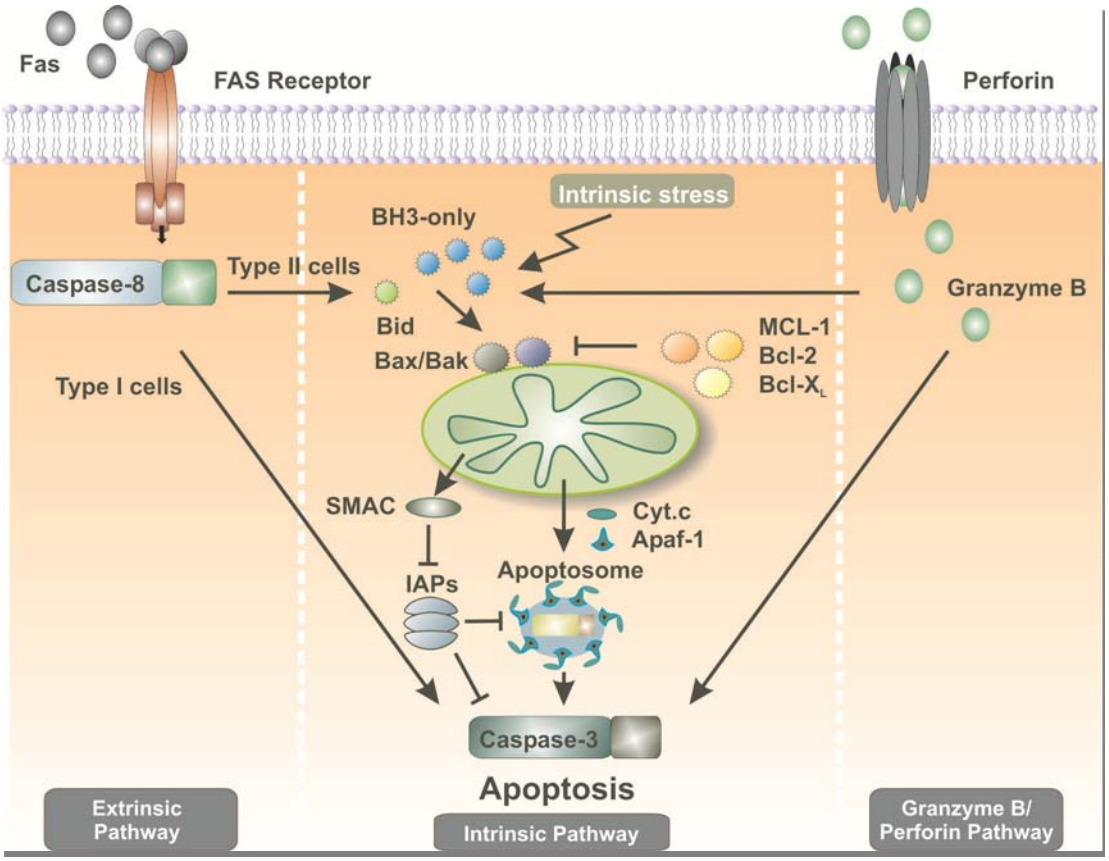


Fig. 1: The three pathways of apoptosis initiation. Apoptosis can be mediated via three pathways, the extrinsic or death receptor mediated pathway, the intrinsic or mitochondria-mediated pathway and the Granzyme B/Perforin pathway. Binding of death ligands to their specific death receptor leads to the activation of the extrinsic pathway by forming a complex where caspase-8 gets processed and activated. Activated caspase-8 can either directly cleave and activate caspase-3 in Type I cells or in Type II cells it can initiate the intrinsic pathway by cleaving Bid, a BH3-only domain protein. The intrinsic pathway can also be initiated by other BH3-only proteins (such as Bid, Bim, Bmf that get activated in response to specific stress signals in the cell. This leads to the activation of Bax/Bak which triggers mitochondrial membrane permeabilization causing the release of pro- and anti-apoptotic factors into the cytosol. These factors form the apoptosome consisting of cytochrome C, Apaf-1 and caspase-9 to trigger caspase-3 activation. This process can be inhibited by antiapoptotic Bcl-2 proteins like Mcl-1, Bcl-2 or Bcl-X_L. Smac released by the mitochondria is required to reverse the inhibition of the caspase activation by the IAP complexes in the cytosol. Finally, in the Granzyme B pathway, perforin and granzyme B are released by cytotoxic T-lymphocytes when they recognize antigen bearing cells. Granzyme B can enter the plasma

membrane with the aid of perforin, and once inside the cell it can directly activate caspase-3 or initiate the mitochondrial step described above.

Extrinsic Pathway: In the extrinsic pathway, apoptosis is initiated when death ligands bind to a transmembrane receptor on the cell surface. The death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily [63]. Some of the well known receptors and their ligands are FasL/FasR, TNFα/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. In the extensively characterized FasL/FasR and TNFα/TNFR1 systems, clustering of the receptors occurs together with the binding of the homologous trimeric ligand [64]. Consequently, cyctoplasmic adapter proteins get recruited to the receptor/ligand complex through their death domains. The binding of TNFα to the TNF receptor, with a simultaneous block in protein synthesis, results in the binding of TRADD (TNF receptor 1-associated protein) with FADD (Fas-Associated protein with Death Domain) and RIP [65,66]. Binding of TNFα to TNFR1 can lead to the activation of nuclear factor κB (NFκB) when protein synthesis is not blocked [67]. Procaspase-8 is then recruited to the complex, where it binds to FADD through dimerization of the death effector domain. This complex is called the Death Inducing Signalling Complex, DISC. Procaspase-8 gets autocatalytically cleaved and activated at the DISC, via a proximity induced activation model [68]. At this stage death receptor mediated apoptosis can be inhibited by a protein called FLICE/caspase-8 Like Inhibitory Protein (c-FLIP) which can bind to FADD and caspase-8 [69]. In healthy tissues, decoy receptors, closely related to TRAIL, are expressed to compete with the death receptors for binding with the ligand [70]. In Type I cells, the amount of activated caspase-8 is high enough to directly activate caspase-3 which leads to apoptosis. However, in Type II cells, FADD recruitment to the DISC and the subsequent generation of active caspase-8 is too little to directly cleave caspase-3 for apoptosis [71]. In these cells, Bid gets activated by caspase-8 and this in turn activates the mitochondrial pathway to initiate apoptosis [72]. Blocking of the mitochondrial pathway, for example by overexpression of Bcl-2 or in absence of Bax/Bak, results in blocking of apoptosis in Type II cells but not in Type I cells.

Intrinsic pathway: A diverse range of non-receptor mediated stimuli can also elicit apoptosis in a cell. These include cellular stresses like radiation, toxins, hypothermia or

infections. The intrinsic pathway can also be initiated directly by the absence of certain growth factors, hormones or cytokines which leads to a failure in the suppression of the death pathways. Each of these stimuli activates a specific BH3 only protein that acts as a sensor to that cellular stress [73]. The BH3 only proteins Bid, Bad, Bim, Bik, Noxa and Puma belong to the Bcl-2 family and are characterized by the Bcl-2 homology domain 3. As described in detail below, the Bcl-2 family of proteins is extensively involved in regulating the intrinsic pathway of apoptosis.

The active BH3-only proteins lead to the activation of Bax/Bak and this process is inhibited by the antiapoptotic Bcl-2 members like Mcl-1. Bax/Bak are responsible for the mitochondrial outer membrane permeabilization causing the release of apoptogenic factors including cytochrome *c* and SMAC into the cytosol. Cytochrome *c* binds and activates the cytosolic Apaf-1 and procaspase-9 forming a complex called apoptosome [74]. Active caspase-9 can then activate caspase-3.

Perforin/granzyme Pathway: In the Granzyme B pathway, perforin and granzyme B are released by cytotoxic T lymphocytes when they recognize antigen bearing cells. Granzyme B is exophytically released through the plasma membrane with the aid of the transmembrane-pore forming protein perforin. Once inside the cell it can directly activate caspase-3 or initiate the mitochondrial step described above.

Granzyme B can cleave proteins at aspartate residues and thus activate procaspase-10 and can cleave factors like Inhibitor of Caspase Activated DNAse (ICAD) [75]. Granzyme B can also initiate the mitochondrial pathway by specific cleavage of Bid but also directly activate caspase-3 inducing apoptosis [76].

1.2.4 Bcl-2 family of proteins

The Bcl-2 (B-cell lymphoma-2) gene was discovered at the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas [77,78]. It was observed that overexpression of the gene strongly inhibited cell death by apoptosis, which is a prominent tumour suppression mechanism. Since then it has been found that the Bcl-2 family of proteins are required for a range of apoptotis related activities including developmentally programmed cell death, tissue turnover and host defence against pathogens.

There are at least 20 mammalian Bcl-2 family members, all of which share at least one conserved Bcl-2 homology (BH) domain [79]. The Bcl-2 family can be broadly divided

into three groups based on their role in either apoptosis activation or inhibition. The first group is characterized by the presence of BH domains BH1-4. This group comprises all anti-apoptotic members including Bcl-2, Bcl-$_{XL}$, Bcl-W, Mcl-1, Bcl-B (also known as Bcl-2L10) and A1. A second group contains proapoptotic members which have the domains BH1 -3 including Bax, Bak and Bok. A third group contains also proapoptotic members, but with only the BH3 domain. Members of this group are Bid, Bad, Bik, HRK, Bim, Bmf, NOXA and PUMA which can bind and regulate the anti-apoptotic Bcl-2 proteins to promote apoptosis.

BH3 only proteins

BH3-only proteins are defined as having homology to the core Bcl-2 family members in only the BH3 domain. These proteins act as sensors to certain cellular stresses, in response to which they get activated and transduce the apoptotic signal to activate Bax and Bak which in turn can activate the mitochondrial pathway [80]. Bim is required for the removal of auto-reactive B- and T-cells and thus has a role in preventing autoimmunity [73,81]. It is also induced by growth factor deprivation by the class O forkhead box transcription factor-3A (FOXO3A) or by the transcription factors CEBP-α (CCAAT-enhancer binding protein-α) or CHOP (CEBP homologous protein) in response to endoplasmic reticulum (ER) stress [82,83]. Puma and Noxa are transcriptionally up-regulated by p53 in response to DNA damage [84,85]. Puma is also required for apoptosis induction in lymphocytes induced by cytokine withdrawal, or treatment with glucocorticoids, phorbol ester, or staurosporine [86,87]. Bad is activated by loss of phosphorylation in mammary epithelial cells in the absence of epidermal growth factor (EGF) while Bid is required for apoptosis of fibroblasts and hepatocytes induced by death receptors [88,89]. Bmf has been shown to have a role in detachment induced apoptosis, termed anoikis in certain cell types. Bim and Bmf are initially associated with the microtubules and the actin cytoskeleton respectively via interaction with a Dynein Light Chain (DLC) and their release can induce apoptosis [90].

In an elegant study, Wei *et. al.* showed that cells lacking both Bax and Bak and not cells lacking only one of these, are fully resistant to cytochrome *c* release and apoptosis induced by tBid [91]. Similar results were reported by Zong *et. al.* who showed that activated BH3 only proteins could not induce apoptosis in the absence of Bax and Bak [92]. Thus it

became clear that Bax and Bak are required for apoptosis induction and lie downstream of the BH3 only proteins in the pathway.

Bax and Bak

Bax mainly resides in the cytosol of healthy cells as a soluble monomeric protein. In response to apoptotic stimuli, Bax undergoes conformational changes at both its N- and C-terminus and translocates to the mitochondrial outer membrane where it forms large oligomeric complexes. In contrast, Bak is constitutively associated with the outer mitochondrial membrane, where it changes its conformation in response to apoptotic stimuli and also forms oligomeric complexes [93,94,95]. Once the Bax and Bak oligomers are formed, mitochondrial permeabilization occurs, culminating in the release of proapoptotic factors into the cytoplasm (reviewed in [96]). Simultaneously with cytochrome *c* release or immediately before, Bax and Bak induce mitochondria to fragment into more numerous and smaller units suggesting that the Bcl-2 family could have a role in mitochondrial division processes [97].

It is known that the Bax/Bak activation is controlled by other members of the Bcl-2 family, though the exact mechanism remains unclear. Huang *et. al.* proposed a model in which all BH3-only proteins can bind Bcl-2 pro-survival proteins and a select few such as Bim, tBid, and possibly Puma also have the capacity to directly bind and activate Bax and Bak. Bax and Bak cannot be activated in the absence of the "direct activator" group of Bim, tBid or Puma. However, other BH3 only proteins called the "derepressors" (Bad, Bik, Hrk, Noxa) that cannot activate Bax and Bak on their own, can induce apoptosis by binding to the pro-survival proteins and freeing the direct activators [98].

It is known that some of the BH3 only proteins such as Bim, Puma are more potent apoptosis inducers compared to other members like Bad or Noxa. Studies measuring the affinity of peptides corresponding to the BH3 domains of the established mammalian BH3-only proteins to their Bcl-2 pro-survival partners showed that Bim and Puma bind with high affinity to all pro-survival members, while the others have more restricted binding profiles [99]. These results suggested that each of the Bcl-2 prosurvivial proteins might have a unique role in antiapoptosis, and that each of these different classes must be neutralized to induce apoptosis. This was further confirmed when it was observed that co-expression of BH3-only proteins with complementary binding profiles, such as Noxa

which neutralizes Mcl-1 and A1, and Bad which neutralizes Bcl-2, Bcl-x_L, and Bcl-w, induced apoptosis to a similar extent as the expression pan-specific BH3-only protein Bim did [99].

1.2.5 Pro-survival Bcl-2 proteins

The Bcl-2 pro-survival proteins act by directly interacting with Bax and Bak at the mitochondrial outer membrane. Bak forms complexes with both Mcl-1 and Bcl-x_L, by interacting through its BH3 domain, but not other pro-survival proteins such as Bcl-2 [100]. Thus Mcl-1 and Bcl- X_L sequester Bak in healthy cells and are both important inhibitors of Bak activation and subsequent mitochondrial damage. This is further confirmed by the fact that Noxa expression does not kill wild type cells because it can only neutralize Mcl-1 and not Bcl-X_L, it efficiently induces apoptosis in cells lacking Bcl-X_L. On the other hand, Bax was observed to bind to Bcl-2 after Bax translocates to the mitochondria following a confirmational change [101].

1.3 Myeloid Cell leukaemia-1

Myeloid Cell Leukemia sequence 1 (Mcl-1) was identified as an early induction gene during myeloblastic cell differentiation. Mcl-1 is one of the most important anti-apoptotic proteins preventing inappropriate activation of Bax and Bak in healthy cells [102]. Mcl-1 is critically required during embryonic development with its deficiency resulting in peri-implantation embryonic lethality [103]. Mcl-1 is also required for the survival of hematopoietic stem cells and in the development of B- and T- lymphocytes [104,105].

Mcl-1 contains a C-terminal transmembrane (TM) domain that results in localisation of Mcl-1 to various intracellular membranes, most notably the outer mitochondrial membrane [106]. There it is known to sequester Bak and maintain it in an inactive state in healthy cells. Because of its strong anti-apoptotic effect, Mcl-1 is required to be removed or neutralized for apoptosis induction. It is degraded in a proteasome dependant manner during apoptosis induction by genotoxic agents like UV radiation or by over-expression of BH3 only proteins. When Mcl-1 degradation is blocked using inhibitors, the apoptosis rate is decreased [99,107].

The expression of Mcl-1 is tightly regulated because of its vital role in cell survival and apoptosis control. Several survival and differentiation signals like cytokines and growth

factors regulate Mcl-1 levels in the cell [108]. Survival pathways like Mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 (PI3K) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) dependant pathways have all been implicated in controlling of Mcl-1 transcription, acting via specific transcription factor response elements in the Mcl-1 promoter [108,109]. However, Mcl-1 expression as well as function is also controlled by posttranslational modifications including direct phosphorylation [110].

Recently, it was shown that HIF-1α has an anti-apoptotic effect that is mediated via Mcl-1 expression [111,112]. It was seen that the transcription factor, HIF-1 controls the expression of Mcl-1 during hypoxia. When the regulatory subunit of HIF-1 was downregulated by silencing with RNAi, the mRNA levels of Mcl-1 were also reduced. It has also been shown that the Mcl-1 gene promoter has a hypoxia responsive element (HRE) that can bind HIF-1 in vitro [111].

1.4 Hypoxia Inducible Factor-1

Hypoxia Inducible transcription Factors, (HIF) are a family of transcription factors that are crucial mediators of the oxygen signaling pathways in a cell. These are heterodimeric, consisting of an oxygen-sensitive alpha subunit (HIF-α) and a constitutive beta subunit (HIF-β). The HIFs facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of genes that control glucose uptake, metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis [113].

HIF-1 was the first member of the HIF-1α family to be discovered - originally characterized as a biologic O_2 sensor that enables the organism to adapt to hypoxia [114]. HIF-1 has emerged as the most important regulator of cellular response to hypoxia since it is ubiquitously expressed and induces the expression of many hypoxia-inducible genes. At the cellular level, HIF-1 induces the expression of virtually all glycolytic enzymes, genes involved in erythropoiesis, pro-angiogenic genes, such as vascular endothelial growth factor (VEGF) and anti-apoptotic genes including *mcl-1* and *cIAP-2* [112,115,116].

In its active form, HIF-1 is a heterodimer composed of an α subunit (100–120 kDa) and a β subunit (91–94 kDa). While HIF-1β is permanently present in cells under all oxygen conditions, HIF-1α is virtually undetectable in normal oxygen conditions, since it gets rapidly degraded by the proteasome. For the HIF-1 transcriptional complex to be

functional, HIF-1α levels must be induced. Under hypoxia, HIF-1α gets stabilized and binds to HIF-1β in the nucleus to form the HIF-1 transcription complex. Interestingly, it has been shown that HIF-1 activity can be increased through phosphorylation. HIF-1α is directly phosphorylated by p42/p44-mitogen-activated protein kinases (p42/p44-MAPK). Activation of the p42/p44-MAPK pathway leads to increased transcriptional activity of the HIF-1 complex and downstream gene activation [117].

1.4.1 HIF-1α and anti-apoptosis

Under severe hypoxic conditions some cells are irreversibly damaged, whereas mild hypoxia can lead to cells adapting to the stress and becoming resistant to injury [118,119,120]. Cells that become adaptive to hypoxia are more resistant to apoptosis and less responsive to cancer therapy [121]. The anti-apoptotic effect of hypoxia arises due to the increased expression of pro-survival factors in the hypoxic cells. Dong et *al*. reported that cells grown under hypoxic conditions were resistant to staurosporine induced apoptosis. It was observed that the hypoxia induced increased levels of cIAP-2 in the cells, which was required for the apoptosis inhibition [122].

The anti-apoptotic effect of HIF-1α was also demonstrated by Liu et. al., who reported that HIF-1α regulated the levels of Mcl-1 in airway epithelial cells. The cells were susceptible to apoptosis induced by anoxia/reoxygenation when either HIF-1α or Mcl-1 levels were decreased by RNAi [112]. Park et. al. showed that human lung carcinoma A549 cells exposed to hypoxia were significantly protected against TRAIL induced apoptosis [123].

1.4.1 HIF-1α stabilization during Chlamydia pneumoniae infection

It has been reported that *Chlamydia pneumoniae* interferes directly with HIF-1α regulation in the infected cell. During hypoxia, the infection had an additive effect on HIF-1α stabilization and HIF-1 activation. However, during late phase of intracellular *Chlamydia* replication (48 h) HIF-1α was seen to be degraded by the activity of the chlamydial protease-like activity factor (CPAF) secreted into the cytosol of the infected cells. It was also observed that HIF-1α was required for the efficient *C.pneumoniae* replication during hypoxia [124]. These data show that HIF-1α is targeted by the chlamydiae to control host cell metabolism and integrity during their developmental cycle.

1.5 Apoptosis in defence against infections

Apoptosis plays an important role in embryogenesis, development of the nervous and immune systems, and tissue homeostasis. In addition, it constitutes a vital defence mechanism against damaged or infected cells. Cytotoxic T lymphocytes can kill tumour cells or virus infected cells by inducing apoptosis [125]. Apoptosis is induced in infected cells and the resulting apoptotic bodies phagocytosed, thus preventing the spread of infection to neighbouring cells. For example, it has been shown that the programmed cell death of *Mycobacterium avium* infected cells is an important defence mechanism, which acts by sequestering M*ycobacteria* and aids in their killing by activation of newly recruited macrophages [126].

Resistance to apoptosis in the infected cells is therefore important for pathogens, especially those residing inside the cell, to evade the host immune response. Several parasitic pathogens, including viruses and bacteria, modulate host cell apoptosis to escape from the host cell immune response and prolong their stay in the host. Bacteria like *Salmonella, Shigella* and *Yersinia* spp., defend themselves from host macrophages by inducing apoptosis [127]. Others like *Helicobacter, Neisseria, Staphylococcus* and *Listeria* spp. induce cell stress by bacterial toxins or effector proteins, resulting in apoptosis of the host cell [128,129,130,131].

1.6 Chlamydia and apoptosis

Obligate intracellular bacteria like *Rickettsia* spp. or *Chlamydia* spp. confer resistance to the host cell against a variety of apoptotic stimuli, including the action of cytotoxic T-cells [132,133]. The bacteria are thus able to maintain a long term relationship inside the host cell. In case of *Chlamydia spp.*, this could help the bacteria to complete its replication cycle inside the cell, at the end of which numerous elementary bodies are produced and released to infect other host cells. *Chlamydia* can sometimes develop long term chronic infections that could contribute to diseases like atherosclerosis or cancer. Protection of the host cell against apoptosis would acquire greater significance for the bacteria under such conditions. It is not surprising therefore, that persistently infected cells have also been shown to be potently resistant to various forms of apoptosis [134,135].

Although apoptosis inhibition by *Chlamydia* has been widely reported, there have also been reports of induction of apoptosis by *Chlamydia* [136,137,138]. However, subsequent studies have confirmed that *Chlamydia* has a predominantly anti apoptotic effect and that the "*Chlamydia* induced cell death" is different from apoptosis, although some of the morphological features may be similar [139,140,141]. In the background of the block in the apoptotic pathways during infection, this cytotoxicity might be the result of cell stress and damage mediated by the host innate immune reaction or by the bacteria itself. The presence of a bacterial inclusion in the cell would inevitably cause damage and stress in the cell, and it is not surprising that some cells die under the circumstances- the cell is after all still mortal, and although it cannot be forced to commit suicide, it can still be murdered!

Chlamydia has evolved various strategies to survive intracellularly, whereby it not only prevents the detection of the host cell by the immune system, but also protects it from destruction by the immune mechanism in case of recognition. The bacteria can down-regulate the expression of MHC Class I and II molecules, thus avoiding the recognition of the host cell by CD^{+} T cells [142],[143]. *Chlamydia* further protects the host cell against cytoxic immune response, by its remarkable ability of making the cell strongly resistant to apoptosis.

Apoptosis resistance has been attributed to all major chlamydial species viz. *C. pneumoniae*, *C. trachomatis*, *C. psittaci, C. muridarum*, as well as *C caviae,* [133,144,145,146]. Of these *C. trachomatis* and *C. pneumoniae* have been widely studied for their strong anti apoptotic effect and relevance to human diseases.

The chlamydial infection protects cells against different forms of apoptosis described above such as the extrinsic, the intrinsic and the granzyme B pathway, mediated by various stimuli including staurosporine, tumor necrosis factor alpha, etoposide, granzyme B/perforin, and UV light [133,144,147]. Initial work characterizing the apoptosis resistance showed that only the inclusion carrying cells were protected, in contrast to any uninfected neighboring cells, suggesting that the effect was conferred by the intracellular bacteria and not through a factor secreted into the medium [144]. Further, chlamydial protein synthesis has been shown to be essential for the effect since treating the cells with the prokaryotic transcription inhibitor rifampin or the translation inhibitor chloramphenicol abrogated the apoptosis inhibition. It was also reported that since *Chlamydia* could block apoptosis even

in the presence of the eukaryotic translation inhibitor cycloheximide, host cell synthesis was not required for apoptosis inhibition [133].

Analysis of the apoptotic pathway in the infected cell showed that there was a block in the release of cytochrome *c* from the mitochondria into the cytosol. Cytochrome *c* is required for the activation of the procaspase-9 through apoptosome formation. Caspase-9 eventually cleaves and activates caspase-3. Expectedly in the infected cells, there was no processing/activation of caspase-9 or caspase-3 [148]. This implied that there was a block in the mitochondrial permeabilization, which in turn is controlled by Bax and Bak. Later studies showed that the chlamydial infected cells failed to achieve the activation of these regulators of mitochondrial permeabilization [149,150]. It was further shown that the infected cells are not resistant to apoptosis in type 1 cells, which do not require the mitochondrial pathway for activation of the effector caspases [151]. With these data it became evident that *Chlamydia* confers a block at the mitochondrial level. This would also explain the resistance to apoptosis initiated through different pathways that collude at the mitochondria.

It has been reported that during chlamydial infection, there is a broad scale degradation of the different BH3-only proteins in the cell. The mRNA levels of these proteins were not affected, indicating that the down-regulation was at the protein level. The Chlamydial protease-like activity factor (CPAF) was shown to be responsible for targeting those active proteins with an exposed BH3 domain, for degradation [150,152,153,154], implying that *Chlamydia* destroy most, if not all, active BH3-only proteins. In absence of the BH3-only proteins, death signals cannot be transmitted to the mitochondria, and this could account for the block in apoptosis upstream of the mitochondria.

In an elegant experiment to analyse the extent of apoptosis inhibition in the infected cells, it was noticed that the cytosolic extract from chlamydial infected cells resisted the activation of caspase-3 even on treatment with cytochrome *c* [155]. This indicated that *Chlamydia* infected cells have a block in apoptosis downstream of the mitochondria as well, that would prevent the activation of caspases in spite of cytochrome *c* release. Evidently, *Chlamydia* interfere with the host apoptotic machinery at different levels- not only at the mitochondria, but also downstream of it.

Genetic studies in the infected cells showed that *Chlamydia* indeed widely interferes with the host protein synthesis. Infection with *C. trachomatis* led to the up-regulation of some genes including certain anti-apoptotic genes such as c-IAP2 [156,157,158,159].

Chlamydial infection has been reported to activate certain survival pathways in the cells including the MAPK and the PI3K pathways [160]. The activation of the PI3K pathway in the infected cells has also been shown to sequester the BH3-only protein BAD away from the mitochondria. It was seen that activation of the PI3K pathway led to the phosphorylation of BAD after which it was recruited to the surface of the chlamydial inclusion [161]. This would further increase the resistance to those apoptosis inducers (e.g. staurosporine) that rely on BAD for activating the mitochondrial pathway.

2 Results

2.1 Characterization of apoptosis resistance in cells infected with Chlamydia trachomatis

All major chlamydial species have been reported to inhibit apoptosis. In the present work *Chlamydia trachomatis* was considered due to its relevance to human diseases and also because of the feasibility in working with it in the laboratory.

2.1.1 *C. trachomatis* inhibits apoptosis induced by different stimuli

Cells infected with *Chlamydia* strongly resist apoptosis induced via the intrinsic pathway, induced for example by DNA damage by cisplatin or thapsigargin or the death receptor-mediated pathway induced by TNF-α/CHX Figure 2A. A time lapse microscopy experiment with HeLa cells infected with *Chlamydia* and treated with TNF-α/CHX showed that only the cells carrying inclusions were protected from apoptosis as shown in Figure 2B. Neighbouring cells without a chlamydial inclusion undergo apoptosis on treatment- this can be observed as the rounding up of the apoptotic cells in the image. This suggests that an active growth of *Chlamydia* inside the cell is required for apoptosis resistance, and that no effector secreted into the medium is responsible for protecting cells. The immunoblot in Figure 2B shows the level of apoptosis in the infected cells compared to that in non-infected cells which were also treated similarly with TNF-α/CHX. Cleavage of Poly (ADP-ribose) Polymerase (PARP) occurs during apoptosis induction and was seen only in the non-infected cells treated with TNF-α/CHX.

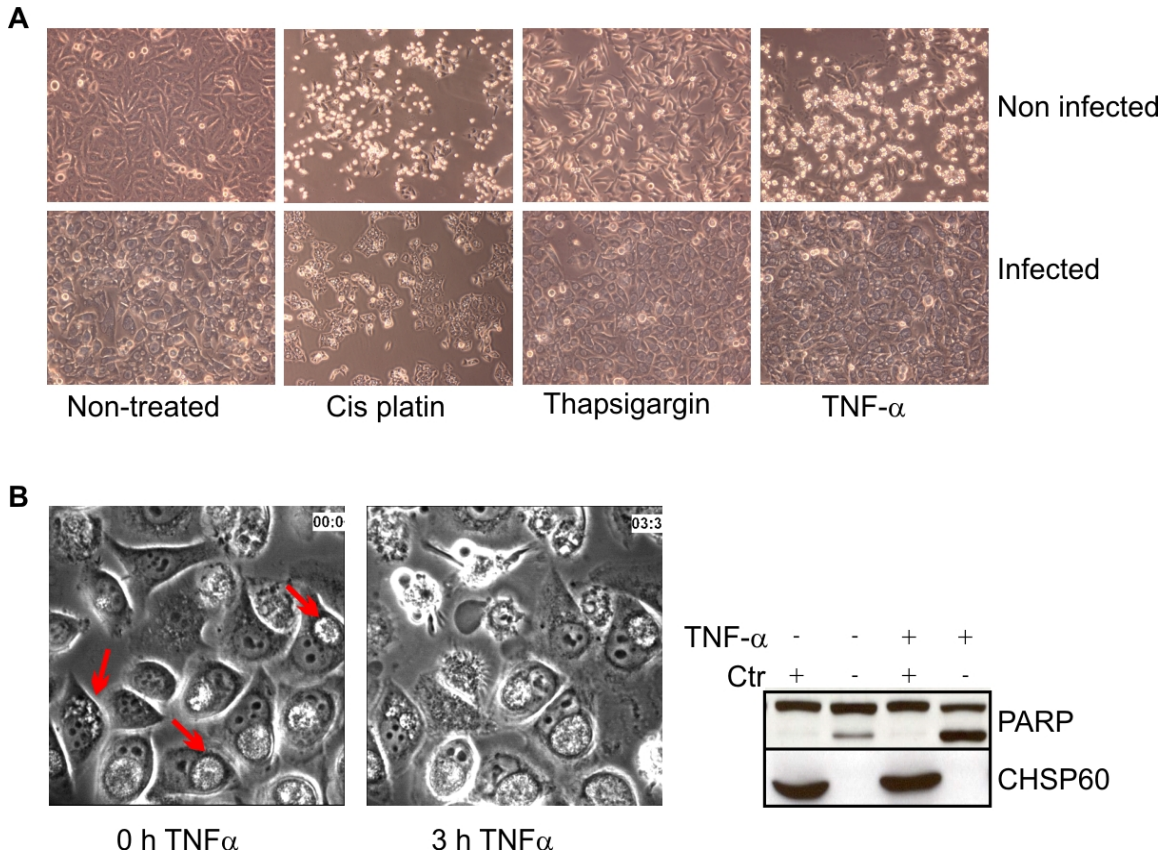


Fig. 2: Cells infected with *Chlamydia trachomatis* resist apoptosis. A: HeLa cells were infected with *Chlamydia trachomatis* (lower panel) or left uninfected (upper panel) and treated with different inducers of apoptosis- Cisplatin, Thapsigargin, and TNF-α/CHX. Phase contrast pictures of the cells show the extent of apoptosis in the infected and non infected cells. The dead cells can be observed as the bright rounded up cells. **B**: Images from time lapse microscopy showing HeLa cells infected with *C trachomatis* and treated with TNF-α/CHX (right picture) at the indicated time points. The arrows point at chlamydial inclusions. The extent of apoptosis was quantified in similarly treated cells by probing for PARP cleavage. Bacterial Hsp60 (CHSP60) was probed to show the extent of chlamydial infection.

2.1.2 Activation of caspase-3 was inhibited in *Chlamydia*-infected cells

During apoptosis, caspase-3 gets cleaved at the aspartic acid residue D175 to yield a small subunit and an N-terminal p19 fragment that contains the prodomain. The prodomain is subsequently cleaved off autocatalytically at the N-terminal fragment yielding a p17 fragment. The fully active caspase-3 is characterized by the presence of these p19 and p17 fragments [162]. During late apoptosis the conversion of the p19 fragment to the p17 fragment is complete as seen in a caspase-3 immunoblot. To check for the activity of

caspase-3 during infection, a time course of infection was done. At each time point apoptosis was induced with TNF-α/CHX and proteins were isolated for immunoblot after 5 h and 10 h post apoptosis induction. Caspase-3 blots revealed that in the uninfected cells, caspase-3 was cleaved to the p19 and p17 fragments as seen after 5 h (Figure 3). After 10 h of apoptosis induction, the p17 fragment is predominantly visible, showing the extensive autocatalytic activation of the cellular caspase-3. However, in the *Chlamydia* infected cells, the generation of the p17 fragment after apoptosis induction was observed to be inhibited. The block in caspase-3 activation appears with the growth of the chlamydial inclusion in the cell around 20 h post infection. After 30 hours of infection, there is no appearance of the p17 fragment even after 10 hours of apoptosis induction. This shows that *Chlamydia* render a robust block in the caspase-3 activation during apoptosis induction.

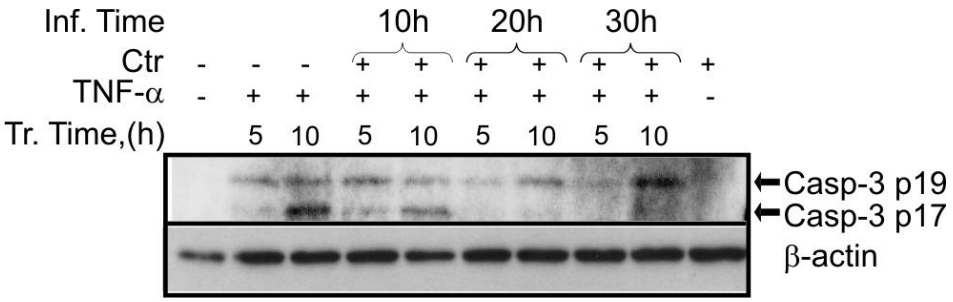


Fig. 3: Immunoblot of Caspase-3 processing during apoptosis induction. HeLa cells were infected with *C. trachomatis (Ctr)* for 10, 20 or 30 h (Inf. Time) or left uninfected and treated with TNF-α/CHX for 5 or 10 h (Tr. Time). Cleavage of caspase-3 to the full active p17 – fragment was abrogated in infected cells 20 h post infection.

2.1.3 Caspase-8 was processed in *Chlamydia* infected cells upon apoptosis induction

HeLa cells were infected with *Chlamydia trachomatis* or left uninfected and apoptosis was induced with TNF-α/CHX at various time points of infection. Procaspase-8 (p60) gets activated by autocatalytically cleaving to a p43/41 proform which is further processed to the active p18 fragment. As is seen Figure 4A the processing of caspase-8 was not altered by the chlamydial infection suggesting that the apoptotic block mediated by *C. trachomatis* does not occur at the step of the initiator caspases.

Bid gets activated by cleavage to a truncated form, called tBid, by active caspase-8 during apoptosis induction by TNF-α/CHX. To check if Bid gets cleaved in *Chlamydia* infected cells an immunoblot was performed testing infected and uninfected HeLa cells which were

induced to apoptosis with TNF-α/CHX or left untreated. As can be seen in Figure 4B TNF-α/CHX treatment leads to the cleavage of Bid in uninfected and infected HeLa cells. This result further confirms that *C. trachomatis* did not inhibit the activation of caspase-8 and did not inhibit the cleavage of Bid.

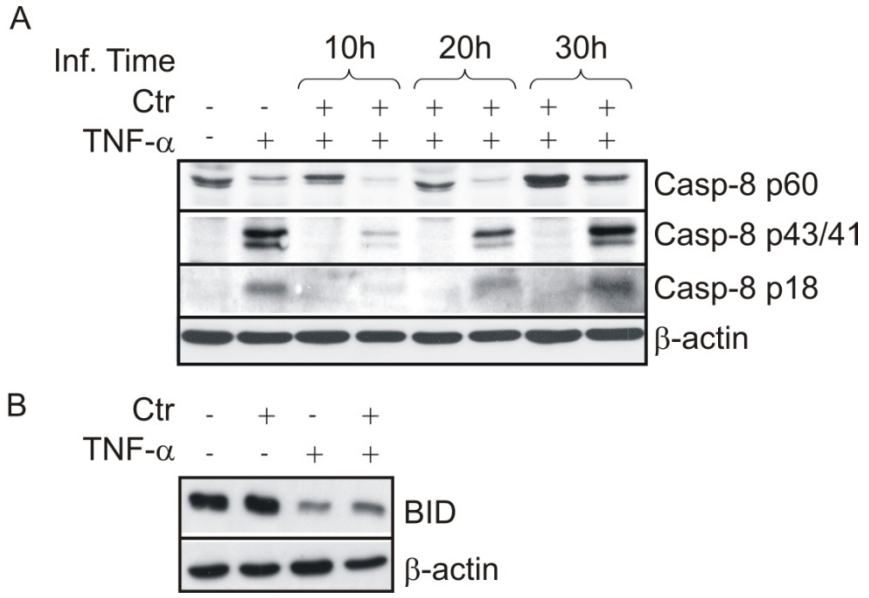


Fig. 4: Caspase-8 is processed normally during apoptosis induction in infected cells..

A: Cells were infected with *C. trachomatis* (Ctr) for the indicated time points and treated with TNF-α/CHX for 5 h. Full size caspase-8 and the cleaved caspase-8 fragments - p43/41 and p18 were probed by immunoblotting. Caspase-8 was normally processed in infected cells. **B:** Infected and non infected cells were treated with TNF-α/CHX and probed for levels of unprocessed BID. Only the proform of Bid was detected in the immunoblot which was reduced in uninfected (third lane) and infected (Ctr, fourth lane) cells induced to apoptosis (upper blot). The lower blot shows the equal loading control.

2.2 The Inhibitor of Apoptosis Protein – complexes are required for apoptosis resistance in Chlamydia trachomatis infected cells

It was observed that *Chlamydia* infected cells have normal caspase-8 activity upon treatment with TNF-α/CHX. However, there was a block in the complete processing of caspase-3 as was seen by the accumulation of the caspase-3 p19 fragment in Figure 3. This suggests a possible involvement of the Inhibitor of Apoptosis Proteins (IAPs), which have been known to directly bind and inactivate the caspases.

2.2.1 cIAP-2 expression is up-regulated during an infection with *C. trachomatis*

HeLa cells were infected with *C. trachomatis* for 24 h or left uninfected and the mRNA expression of different IAPs was determined by quantitative real-time PCR (Figure 5A)[1]. It was observed that the mRNA level of cIAP-2 was increased considerably, whereas the levels of cIAP-1, XIAP or survivin did not change noticeably post infection.

With immunoblot analysis it was seen that the protein levels of cIAP-2 and survivin were increased significantly, whereas the levels of XIAP and cIAP-1 remained more or less constant Figure 5B. Further time course studies revealed a time dependent up-regulation of cIAP-2 and survivin 24 h post infection Figure 5C. Significantly, the increase in the levels of cIAP-2 corresponds to the apoptosis resistance in the infected cells as the infection progresses.

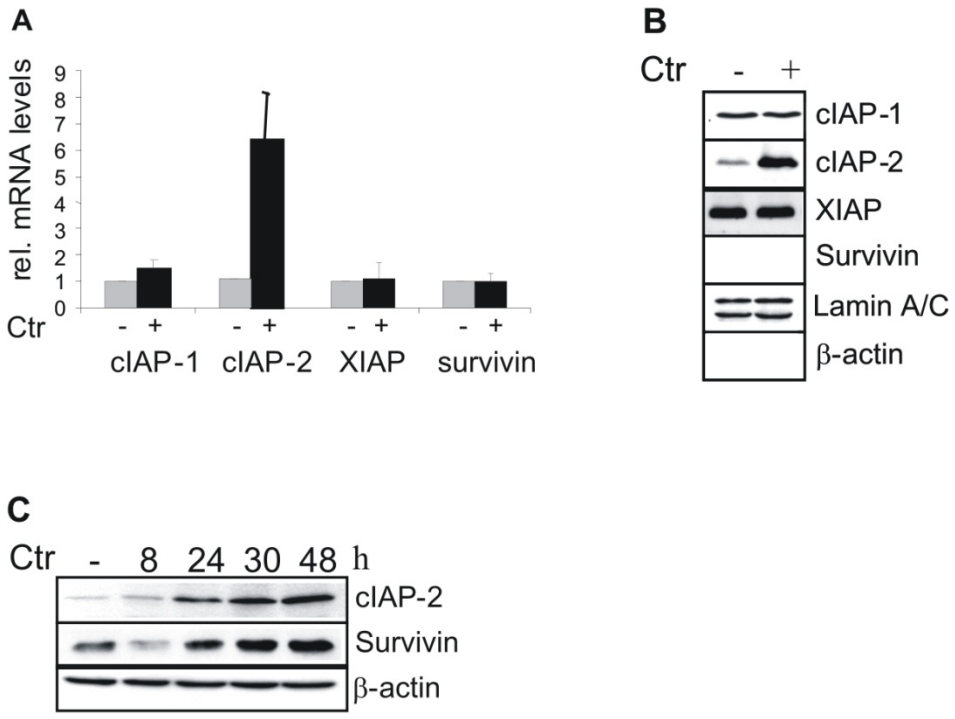


Fig. 5: cIAP-2 expression is up-regulated after chlamydial infection. HeLa cells were infected with *C. trachomatis* (Ctr) or left uninfected. **A**: The mRNA expression was determined 24 h post infection. **B**: Protein levels of different IAPs 24 hrs post infection, cIAP-2 and survivin are heavily up-regulated 24 h post infection. **C**: A time course of infection revealed an up- regulation of cIAP-2 and survivin protein 24 h post infection.

[1] Work performed by Rajalingam et. al. in the Rudel group, [163].

2.2.2 IAPs are required for apoptosis resistance in the infected cells

In order to elucidate the importance of the up-regulation of cIAP-2 in *C. trachomatis* infected cells cIAP-2 was knocked down with small interfering RNAs (siRNAs). HeLa cells were transfected with two different siRNAs against cIAP-2 (sicIAP-2) and a control siRNA against luciferase (siLuc) and infected with *C. trachomatis* or left uninfected. 24 hours post infection, the cells were treated with TNF-α/CHX to induce apoptosis. Apoptosis was measured by estimating the cleavage of caspase-3 by immunoblotting.

It was observed that the knock down of cIAP-2 sensitized the infected cells to apoptosis. Interestingly, the caspase-3 immunoblot showed that in the absence of cIAP-2, the processing of procaspase-3 into the cleaved caspase-3 fragments – p19 and p17 – occurred normally in the infected cells (Figure 6). The relative levels of the p19 and p17 fragments give an idea of the extent of apoptosis in a cell. Therefore, these levels were determined by densitometric analysis in the cIAP-2 knock down samples and compared to in the control samples. In infected control cells, there was a 58 % reduction in the generation of the p17 fragment as compared to the non-infected cells, upon apoptosis induction. After cIAP-2 knock down, there was only a reduction of 29 % in the p17 fragment generation, when the infected cells are compared to the non-infected cells. This demonstrates a 50 % increase in the p19 to p17 conversion, upon cIAP-2 knock down, thus hinting at an important role of cIAP-2 in regulating the complete processing of the effector caspase-3.

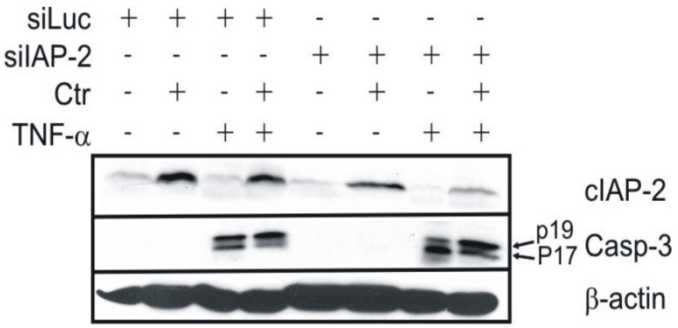


Fig. 6: Caspase-3 is processed normally in Ctr. infected cells with cIAP-2 knock down. Control and cIAP-2 knock down cells were infected (Ctr) and treated with TNF-α/CHX. Levels of cleaved caspase-3 were checked by immunoblotting of the different lysates. The block in the conversion of the p19 to p17 fragment in the infected cells is observed to be reversed in the absence of cIAP-2. Caspase-3 was processed normally in Ctr. infected cells with cIAP-2 knock down.

In a similar experiment, siRNAs against cIAP-1, XIAP and survivin were also designed and the effect on apoptosis induction in the infected cells checked. Surprisingly it was noticed that knocking down of cIAP-1 and XIAP (and not of survivin) lead to a sensitization to apoptosis, even though it had been observed earlier that the levels of these two IAPs are not increased during the chlamydial infection (Figure 7) [1]. The results were confirmed with a different set of siRNAs, to rule out any off-target effects of the first set of siRNAs. These experiments showed that the multi-BIR-containing IAPs viz. cIAP-1, cIAP-2 and XIAP have a role in apoptosis resistance in the infected cells.

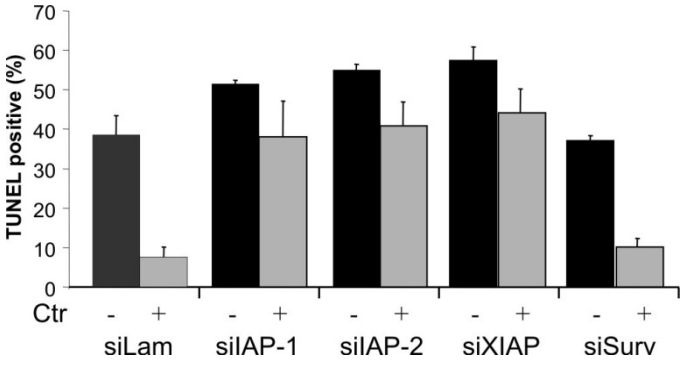


Fig. 7: Knock down of IAPs sensitizes infected cells to apoptosis. HeLa cells with RNAi mediated knock down of IAPs or Lamin control were infected with Ctr. and treated with TNF-α/CHX. The rates of apoptosis were measured by a TUNEL assay. Loss of cIAP-1, cIAP-2, XIAP but not Survivin sensitized the infected cells to apoptosis.

2.2.3 IAPs interact with each other, existing in large heteromeric native complexes

The fact that although only cIAP-2 is overexpressed in the infected cells, but still the cell gets sensitized to apoptosis in the absence of the other IAPs, shows that these proteins might work in a concerted fashion- requiring the presence of each other for full functionality. Previous reports have shown that the IAPs can interact with each other. To confirm the formation of IAP complexes in our system, gel filtration analysis was performed. It was important to exclude the presence of mitochondrial Smac from the sample before the interaction of IAPs could be measured. Smac is known to interact and bind to the different IAPs. This could lead to the appearance of the IAPs in high molecular weight fractions. Smac is localized in the mitochondria of non-apoptotic cells. Therefore,

[1] Work performed by Rajalingam et. al. in the Rudel group, [163].

before gel filtration, the mitochondria were separated from the cytosol by cell fractionation, and only the cytosol was used for further analysis. The cytosol of HeLa cells was isolated by subcellular fractionation and the proteins separated by gel filtration. The different fractions were probed for the presence of certain IAPs by immunoblotting. cIAP-1, cIAP-2 and XIAP were found to be present in fractions containing eluates of around 400 kDa in size (Fig.8). The proteins were also eluted in fractions representing their monomeric forms (around 50-60 kDa). Survivin was mainly detected in a low molecular weight fraction, consistent with its monomeric size (Fig.8).

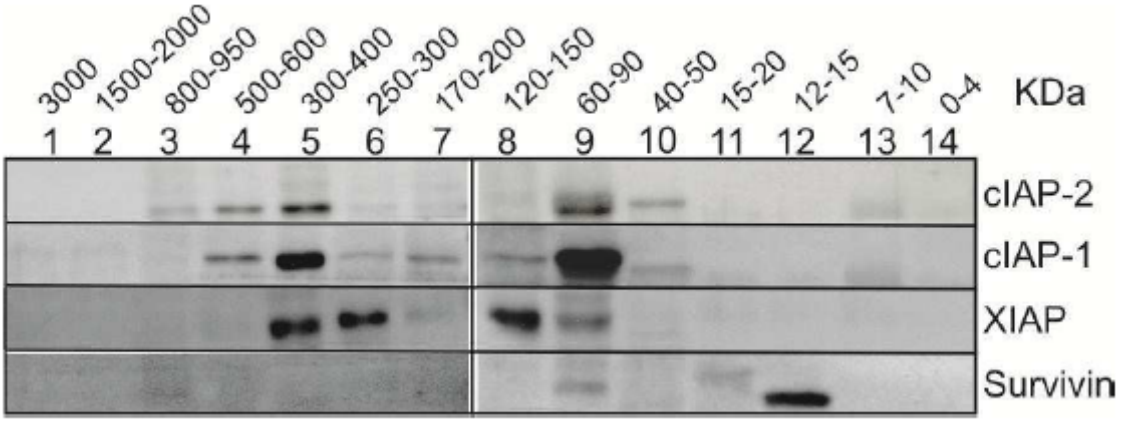


Fig. 8: IAPs are organized in high molecular weight complexes. Subcellular fractions from the cytosol of HeLa cells were separated by gel filtration as described in Materials and Methods section. The presence of IAPs in each fraction was checked by immunoblot analysis. The fraction numbers in the figure are marked with the approximate size of the proteins (and complexes) eluted in the fraction. The numbers on the top represent the fraction number, with the molecular weight range of the proteins eluted.

2.2.4 The formation of IAP complexes is not cell type specific

In order to investigate if the presence of IAP complexes was restricted only to HeLa cells, Hec1b and Jurkat cells were also subjected to gel filtration. The same IAP complexes were also present in Hec1b and Jurkat cells. When the cytosolic fractions of Hec1B or Jurkat cells were analysed by gel filtration, cIAP-1, cIAP-2 and XIAP were observed in high molecular weight fractions similar to HeLa cells, (Figure 9).

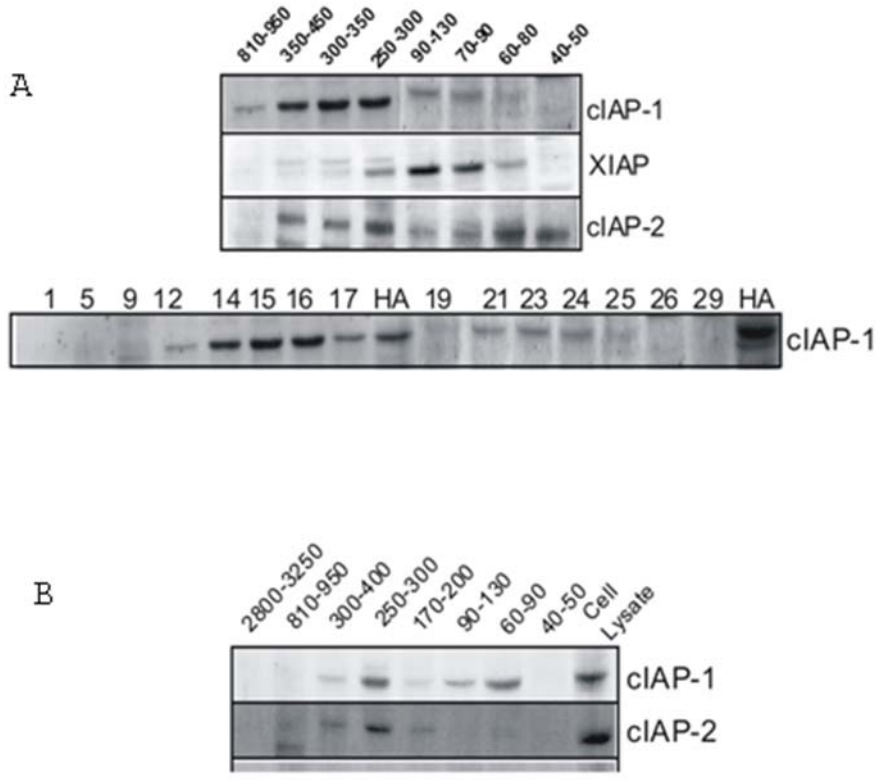


Fig. 9: IAP complexes are detected in different cell types viz. A: Gel filtration studies with Hec1B cells. The lower panel shows the original cIAP-1 immunoblot from the upper panel, which was cut for compiling with the blots for XIAP and cIAP-2. HA refers to whole cell lysate.
B: Gel filtration studies on Jurkat cells. The IAP-IAP complexes are observed in the Jurkat cells also.

2.2.5 Smac and caspase-3 get recruited to the IAP-IAP complex during apoptosis

To check the state of the complexes in apoptotic cells, HeLa cells were treated with TNF-α/CHX for 6 hours and the cytosols from the apoptotic cells isolated and analysed by gel filtration like before. It was seen that lower amounts of IAPs were present in the IAP-containing fractions, suggesting that IAPs get degraded during apoptosis (Figure 10) It was also noticed that a small amount of cIAP-1 was now present in a fraction corresponding to a higher molecular weight complex around 850 kDa in size.

During apoptosis, Smac binds to the IAPs to inactivate them. When the blots were probed for Smac, it was seen to be present in the fractions containing the IAP complexes.

Further, we also found cleaved caspase-3 together with fractions containing the complexes (Figure 10). Together, these data suggest that the IAP complexes have a functional role in

recruiting and inactivating caspase-3. Smac might also bind to the complex, competing with the IAPs for caspase-3 binding.

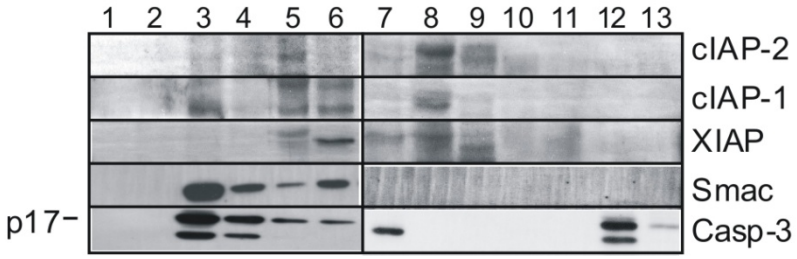


Fig. 10: Smac and Caspase-3 get recruited to the high molecular weight IAP complexes during apoptosis induction. Gel filtration was performed on the cytosol of apoptotic HeLa cells, and the proteins from the fractions TCA precipitated as before. The samples from each fraction were probed for the presence of the IAPs, Smac and cleaved caspase-3.

To confirm that it was not a cell specific response, apoptosis was induced in Jurkat cells with CD95 antibody and the cytosolic proteins analyzed by gel filtration as before. Smac and caspase-3 recruitment to the IAP complex was also seen in Jurkat cells which were induced to apoptosis with the CD95 Ligand. This ligand binds to specific death receptors on the Jurkat cells and induces apoptosis via the FAS/FASLigand pathway.

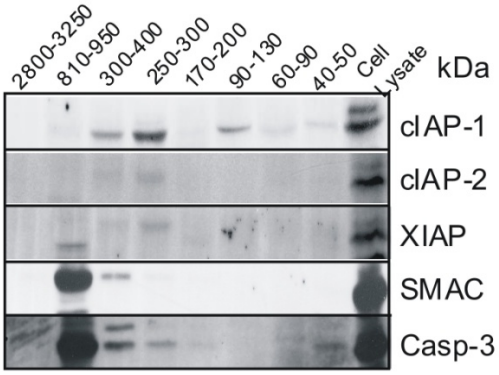


Fig. 11: Immunoblot of gel filtration experiments with Jurkat cells induced to apoptosis. Smac and Caspase-3 are recruited to the IAP complexes upon apoptosis induction in Jurkat cells. Gel filtrations studies on apoptotic Jurkat cells (after treatment with the CD95 antibody) showing the IAPs, SMAC and cleaved caspase-3 in a high molecular weight complex. molecular weight complex.

It was interesting to check if the complex would break down in the absence of any one of the member IAPs. If one of the IAPs was depleted, would the other IAPs still exist in a

complex? A HeLa cell line expressing a permanent XIAP knock down was used and analyzed by gel filtration studies as before. Interestingly, it was seen that the cIAP-1 and cIAP-2 were no longer present in the high molecular weight complex in the absence of XIAP (Fig. 12). This further confirms that the IAPs interact and perhaps regulate the stability of one another. A part of cIAP-2 was now seen in a different fraction, around 850 kDa in size, suggesting its presence in a different complex.

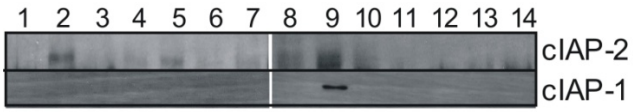


Fig. 12: IAPs are not detected together in high molecular weight complexes in absence of XIAP. Gel filtration was performed on the cytosol from XIAP knock down cells, and the proteins from each fraction were TCA precipitated. Immunoblotting was done to check for the presence of cIAP-1 and cIAP-2 in the different fractions.

To conclude, it was observed that cIAP-1, cIAP-2 and XIAP exist in large native heteromeric complexes in a cell. During apoptosis induction, Smac and caspase-3 got recruited to these IAP complexes. The results were verified in three different cell lines. Further, when XIAP expression was reduced by RNAi, the presence of other members was not detected in a complex

2.3 *BH3 only proteins are not degraded in cells infected with* Chlamydia trachomatis

It has been proposed that the apoptosis resistance in *Chlamydia* infected cells was due to the degradation of the BH3 only proteins in the cells. To check for the degradation of these proteins, an infection time course was carried out and the levels of Bim, Bad, Puma and Bid at the different time points were analysed by immunoblotting. It was seen that the levels remained more or less unchanged during the course of infection (Figure 13). Quantification of the bands was done by densitometric analysis and the results plotted (**Fig. 14**). The results showed that the protein levels of Bid, Bad and Puma remained almost the same during infection time course. The levels of Bim were seen to decrease at 15 h post infection. However, 24 h post infection the Bim levels were seen to be similar to the control cells. Since *Chlamydia* can block apoptosis at 24 h post infection, it is unlikely that the slight Bim degradation (as observed at the 15 h time point) confers the resistance. The decrease in the Bim levels could be because of reversible phosphorylation of Bim at an early phase of infection.

To check if the antibodies were specific for the protein of interest, siRNAs were used to silence the respective genes, and the transfected cells used as a negative control. To compare our infection conditions with other published conditions, the levels of cytokeratin-8 were also determined at different time points by immunoblotting. Cytokeratin-8 is another protein that gets cleaved during chlamydial infection. In fact the degradation of cytokeratin 8 is used as a marker for the chlamydial protease like activity (proposed to be induced by the chlamydial protease like activity factor (CPAF)). It was seen that as observed in other studies, the levels of cytokeratin 8 decreased significantly with the growth of the chlamydial infection (seen by the levels of the chlamydial Hsp60) which rules out major differences between the infection conditions employed as compared to others.

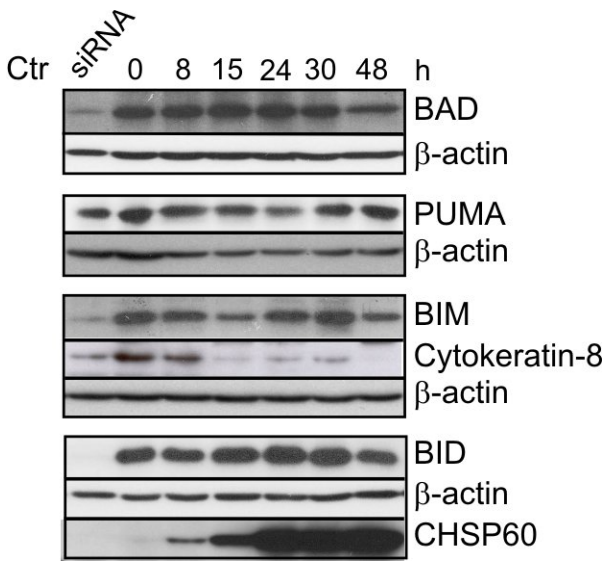


Fig. 13: Immunoblot showing protein levels of BH3 only proteins in a time course of infection in HeLa cells. HeLa cells were infected with *C. trachomatis* for the indicated time points. In the first lane of every blot, a sample with a siRNA mediated knock down of the particular BH3 only protein (BAD, PUMA, BIM or BID) was used as a control to check the specificity of the antibody. Bacterial Hsp60 (CHSP60) was used as an infection marker. The activity of *chlamydial* protease like activity factor, CPAF, was measured by detecting the levels of its substrate- Cytokeratin-8 at the different time points.

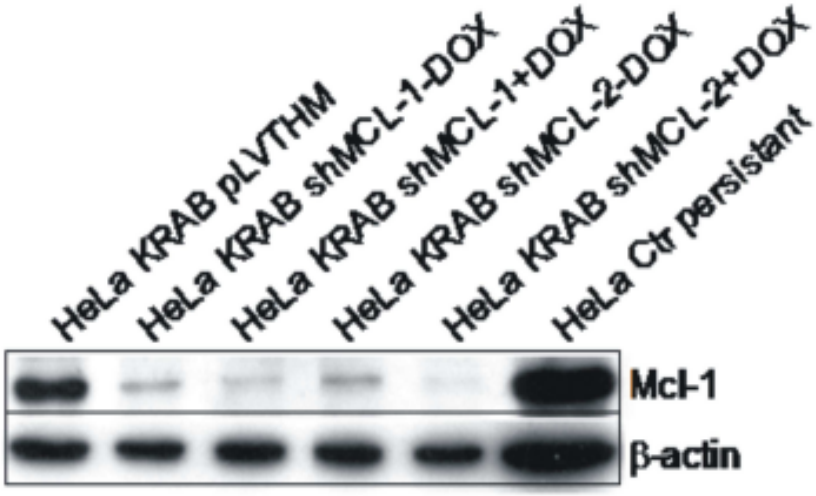


Fig. 14: Densitometric analysis of immunoblots from Fig 13. The intensity for each band was normalized with the respective Actin control. The intensities of bands were determined to be nearly constant for the different BH3 only proteins. 15 h post infection, the levels of Bim were seen to be depleted.

To further confirm the results, HeLa cells were grown on coverslips, infected with *C.trachomatis* and fixed at different time points. The BH3 only proteins were stained with the respective antibodies and the levels at different time points compared by immunofluorescence (Figure 15). As before, antibody specificity was checked by using RNAi for the respective genes. Consistent with the immunoblot analysis it was seen that the BH3 only proteins levels remained more or less constant during the infection time course.

These data show that the degradation of the BH3 only proteins cannot account for the apoptosis resistance in the infected cells. Therefore it becomes apparent that there must be different mechanisms at work in the cell that confer a block in apoptosis.

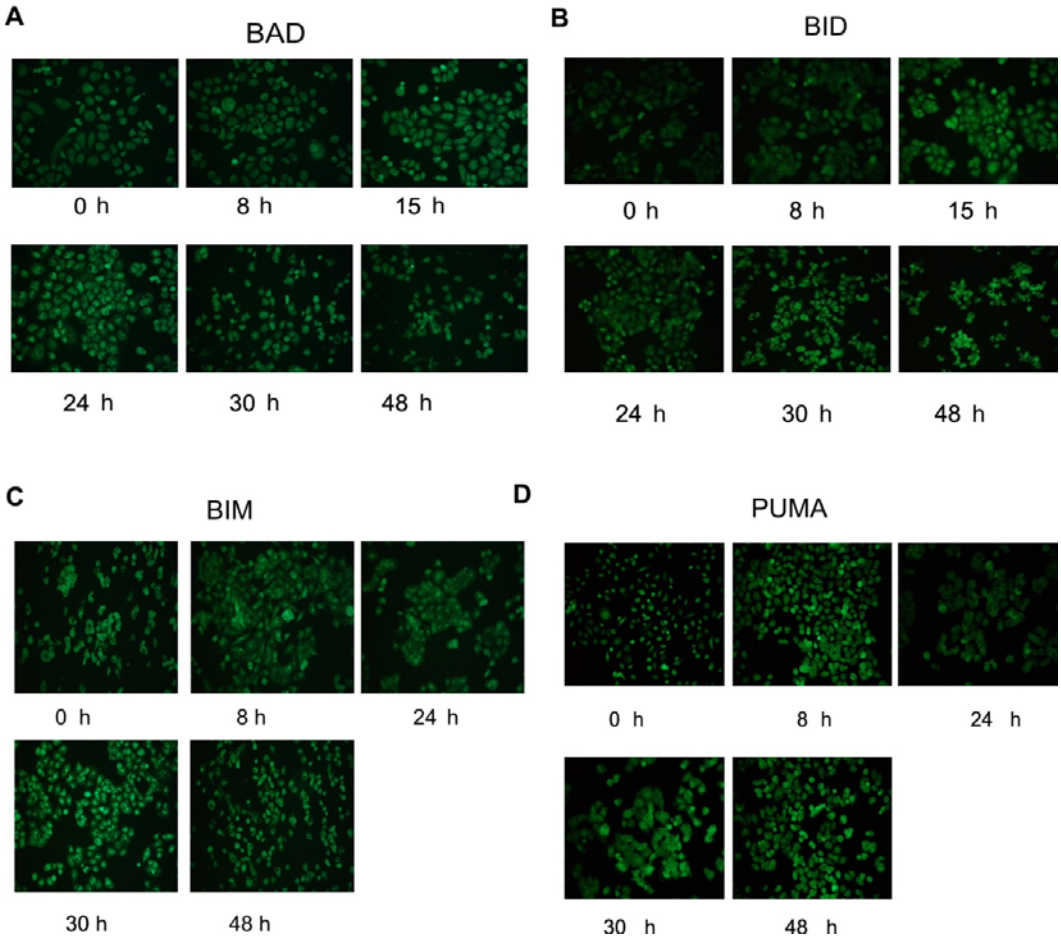


Fig. 15: Levels of BH3 only proteins remain constant during the course of infection. HeLa cells were infected with *Chlamydia trachomatis* for different time points and stained for the BH3 only proteins- Bad, Bid, Bim and Puma.

2.4 RNA interference screen to identify factors involved in infection induced apoptosis resistance

An RNAi screen was designed to determine host cell genes, whose silencing led to the sensitizing of the cells to apoptosis. A custom made siRNA library against 473 genes was employed to target part of the genome related to apoptosis, cellular trafficking and cell signalling. The siRNAs were validated with qRT-PCR analysis to ensure a high level of knock down during the screening process. HeLa cells were grown on a 96 well plate. Three days after transfection of siRNAs to silence gene expression, cells were infected with *Chlamydia* and apoptosis was induced with TNF-α/CHX at 24 h post infection. The analysis was carried out at least three times independently with each siRNA. The nuclei of the cells were stained with Hoechst stain together with the M30 staining to mark the apoptotic cells. An automatic microscope was used to capture images of the stained cells and nuclei in each well of the different 96 well plates, and the numbers counted with a sensitive image analysing software, ScanR. Computations were done to identify statistically significant results- targets whose silencing lead to a significant apoptotic induction in the infected cells, as compared to the control cells[2].

To confirm the results from the automatic microscope and the image analysis software, the acquired images were analysed manually. The nuclei and the apoptotic cells in these samples were counted manually and compared with the results obtained by the software. This was required to rule out any false targets obtained from the image analysis software.

A group of 32 targets was identified with the automated microscopy and image analysis and the results were confirmed manually. The targets are mentioned in Table 1. The most prominent target emerging from the screen was Mcl-1, a member of the Bcl-2 family (Figure 16). Besides Mcl-1, at least six member of the RAS signalling pathway were identified. Though previous reports have shown the activation of the MAPK pathways on *Chlamydia* infection, the identification of these targets clearly implicated the involvement of these pathways in apoptosis resistance. It should be noted that other members of the MAPK signalling cascade might not have been identified because the corresponding apoptosis levels might be lower than the threshold values followed. It is also likely that because of the redundant nature of pathway proteins, knock down of any one protein might not fully block the signalling cascades. In this scenario, the enhanced survival “signal”

[2] The experiments for the screen were performed by Nikolaus Machuy et. al.

upon *Chlamydia* infection would still be transmitted downstream, and consequently the particular protein would fail to get identified in the screen. Pathway analysis of the targets therefore became essential to give a better idea of the mechanism of apoptosis inhibition.

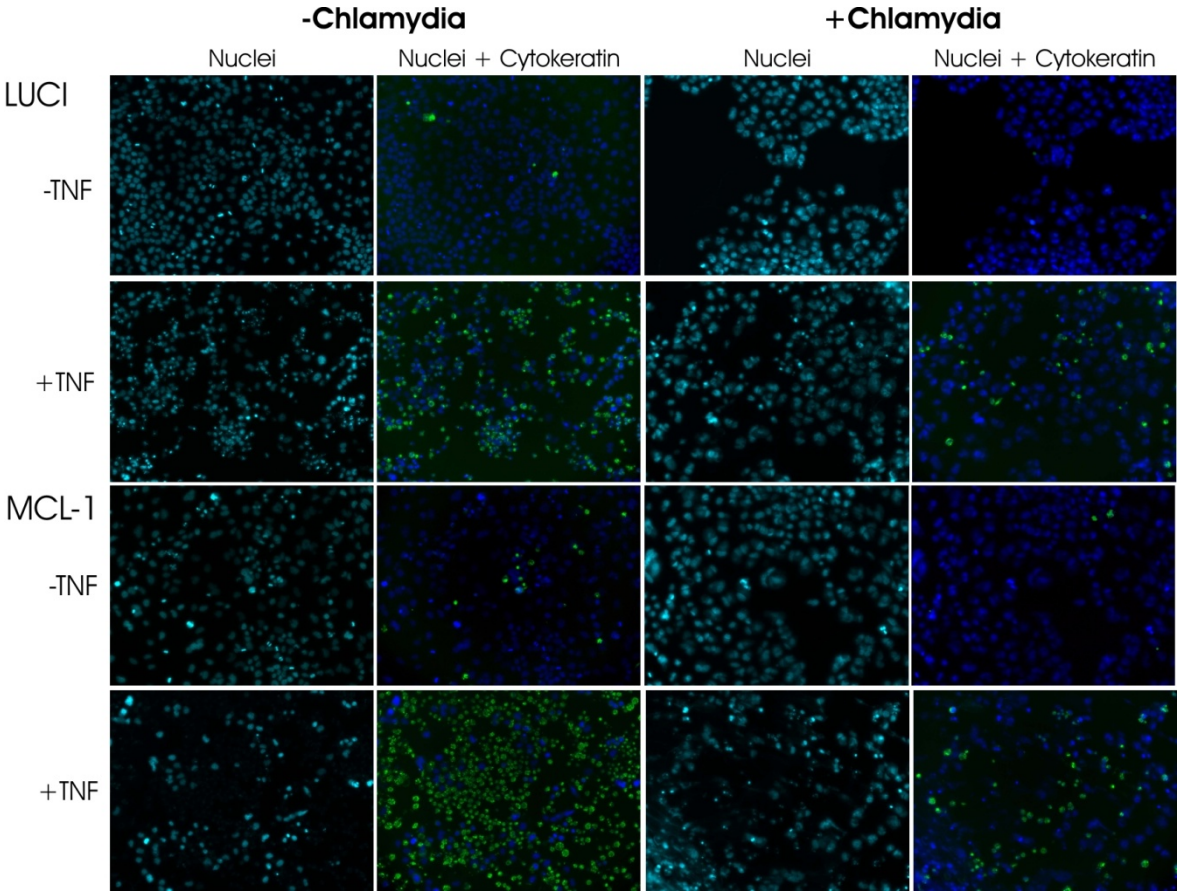


Fig. 16: RNAi screen identifies Mcl-1 as a prominent factor involved in apoptosis resistance in the *Chlamydia* infected cells. Images obtained from the automated microscope showing the siLuci transfected and the siMcl-1 transfected cells in the RNAi screen. The nuclei are stained blue with Hoescht and the apoptotic cells are stained green with the Cytokeratin stain. There is an increase in the apoptosis levels in the siMcl-1 cell population infected with *C.trachomatis*, as compared with the siLuci population as can be seen by the increase in the number of green cells.

2.4.1 Pathway analysis of the targets from the screen implicates HIF-1α as a key regulator of apoptosis resistance

The targets were further analyzed with the specialized software- Ingenuity Pathway Analysis. Interestingly, it was seen that several of the signalling pathways/interactions of

the targets involved the transcription factor Hypoxia Inducible Factor-1 (HIF-1) (Figure 17). The role of HIF-1, and its regulatory subunit HIF-1α was analyzed in detail, as described later.

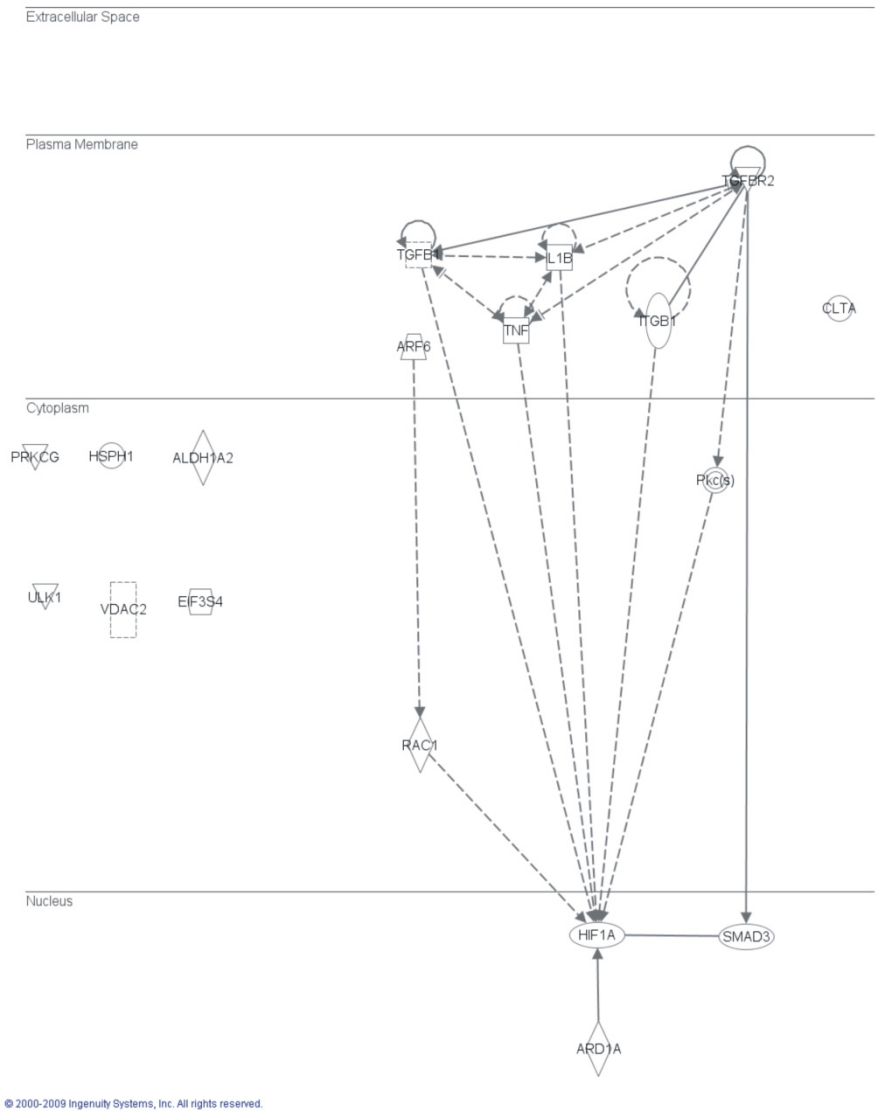


Fig. 17: Pathway analysis identifies HIF-1 as a targets from the RNAi screen. The genes mentioned in the pathways above are Transforming Growth Factor β (TGFB), Interleukin 1 β (IL1B), transforming growth factor, beta receptor II (TGFBR2), Tumor Necrosis Factor (TNF), ADP-ribosylation factor 6 (ARF6), Protein Kinase C (serine) (Pkc (s)), rho family small GTP binding protein (Rac1), Hypoxia Inducible Factor 1 (HIF-1), mothers against decapentaplegic (SMAD3), ARD1 homolog A, N-acetyltransferase (ARD1A).

2.5 Mcl-1 constitutes the prominent block upstream of the mitochondria, to prevent apoptosis in Chlamydia infected cells

Mcl-1 was identified as a major player in apoptosis resistance by the RNAi screen. We next wanted to see how the Mcl-1 expression is modulated during the infection and the effect this has on various stages of the apoptotic pathway

2.5.1 Mcl-1 gets up regulated during chlamydial infection

Consistent with previous reports, it was seen that the mRNA levels of Mcl-1 get up regulated during infection. The up-regulation was seen to occur in a time dependant manner corresponding to the growth of the infection in the cell (Figure 18).

Further, immunoblot analysis showed that the protein levels of Mcl-1 were also significantly increased upon infection. This was also confirmed in the human primary END-1 cells showing that Mcl-1 up-regulation is a general effect of chlamydial infection. A time course of infection revealed an increase in the protein levels of the Mcl-1 after 15 hours of infection.

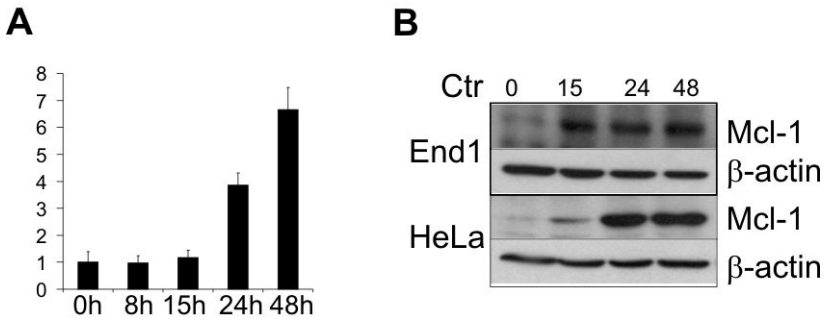


Fig. 18: Mcl-1 expression is up regulated during chlamydial infection. A: HeLa cells were infected with *Chlamydia trachomatis* and q-RT PCR analysis performed for the different time points of infection. The mRNA levels of Mcl-1 increased with the time course of infection. **B**: End1 and HeLa cells were infected with Chlamydia and lysed at different time points post infection. Immunoblot analysis revealed the increase in Mcl-1 at the protein level in both cell lines, with the infection time course.

2.5.2 Mcl-1 is up regulated in a MAPK dependant process

Many of the targets of the screen belonged to the MAPK signalling family. Manual observation of screen raw data showed that the knock down of these members led to

noticeable sensitization of the infected cells to apoptosis, though the sensitization was not as complete as that with Mcl-1. The involvement of the MAPK pathways in the apoptosis resistance was expected since it has already been shown before that chlamydial infection can lead to the activation of MAPK in HeLa cells. Consistent with these results, it was also seen in our group that Ras-GTP could be pulled down from the infected cells. The phosphorylation of ERK, AKT and Raf was also observed after infection [159].

But the question remained as to how exactly these pathways convey the resistance against apoptosis. It is known that MAPK kinase pathways can regulate Mcl-1 expression. It was probable that the chlamydial infection caused the activation of the MAPK signalling pathways resulting in the accumulation of Mcl-1 in the cells.

To check for this, specific inhibitors for the MAPK pathways were employed and the levels of Mcl-1 after infection was checked. Uo126 was used to inhibit MEK-1 and LY294002 was used to block the PI3K pathway. The effectiveness of the inhibitors on the pathway was checked by probing for pAKT and pERK.

The results showed that the Mcl-1 up-regulation was blocked at the protein level, in the presence of both the inhibitors (19). Different concentrations of the inhibitors were used, and the block was seen to be concentration dependent.

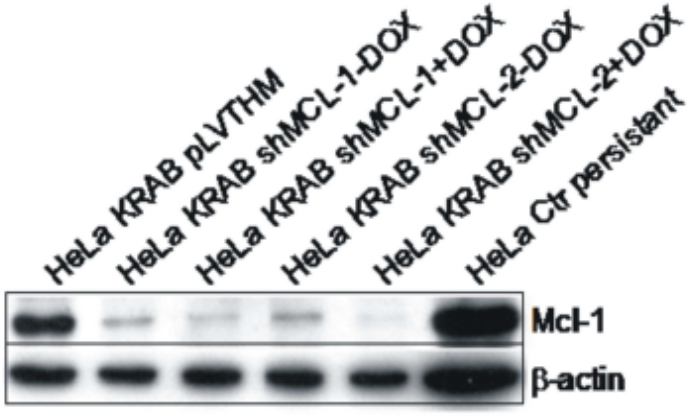


Fig. 19: MAPK pathways are required for Mcl-1 up-regulation in the Ctr. Cells were infected with *Chlamydia* and then treated with different concentrations of the MEK-1 inhibitor UO126 or the PI3K inhibitor LY294002. The cells were lysed 20 h post infection and the samples were probed for the levels of Mcl-1, pAKT, pERK by immunoblotting. The second lane has samples from cells infected with heat inactivated (HI) *Chlamydia*. The infection mediated up-regulation of Mcl-1 was seen to be abrogated in the presence of the inhibitors.

In additional work done in the group, it was seen with Q-PCR analysis that the Mcl-1 up-regulation at the mRNA level was dependent on the MEK pathway, whereas PI3K pathway was required for the protein stability of Mcl-1 in infected cells [159]. Interestingly, it was seen that the inhibitors also blocked the up-regulation of cIAP-2 at the protein level. (The levels of cIAP-2 remained unaltered at the mRNA level).

2.5.3 Mcl-1 is required for resistance to apoptosis in the infected cells

HeLa cells with a permanent Mcl-1 knock down were sensitized to apoptosis when treated with TNF-α after infection. Apoptosis was induced at different time points of infection, and it was seen that after 24 h post infection, the shMcl cell line had near complete PARP cleavage whereas the control cell line had a block in PARP cleavage (Figure 20). The shMcl cell line was the clone-1 as characterized in Figure 21.

Interestingly, after 48 h the cells were resistant to apoptosis even in absence of Mcl-1, confirming that there is a different mechanism at play at the later stages of infection.

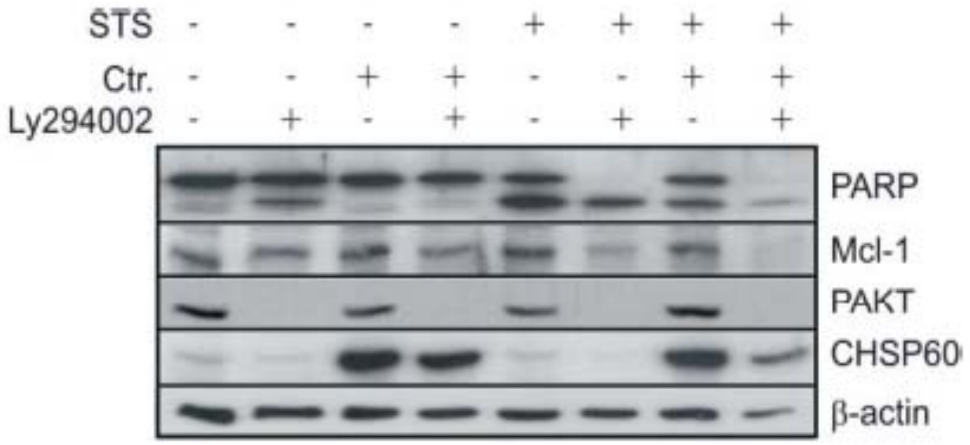


Fig. 20: Silencing of Mcl-1 sensitizes cells to apoptosis during early time points of infection. Control (HeLa cells with empty vector) and shMcl-1 cells were infected and treated with TNF-α/CHX at the different indicated time points of infection. PARP was probed with immunoblotting to measure apoptosis. Bacterial Hsp60 (CHSP60) was used as an infection marker, while Tubulin was used as a loading control. Knock down of Mcl-1 sensitized cells to apoptosis 24 h post infection. However, the cells continued to block apoptosis at 48 h post infection, even in the absence of Mcl-1.

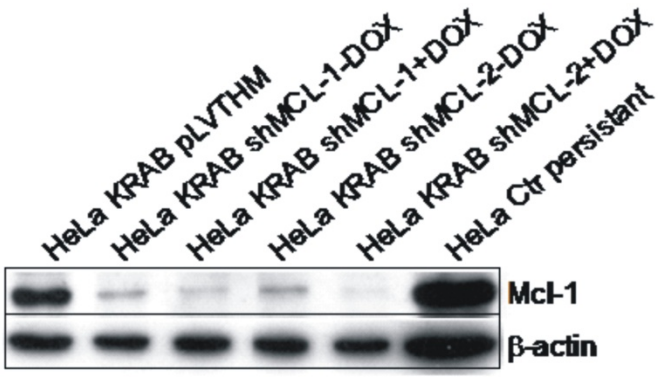


Fig. 21: HeLa cell lines containing vectors for shRNA against Mcl-1 or empty vector (pLVTHM). The vector system imparts a inducible knock down on the cells, upon treatment with Doxycycline (DOX). However, the 2 cell lines were observed to have a significant knock down of Mcl-1 even without DOX treatment, and were used as such in subsequent experiments.

2.5.4 The inhibition of PI3 Kinase sensitized infected cells to Staurosporine induced apoptosis.

The primary human cell line END-1 was sensitized to apoptosis after treatment with LY294002 to reduce the Mcl-1 levels in the cells post infection. The END-1 cells were indifferent to siRNA transfection and therefore the PI3K inhibitor was employed to block Mcl-1 up-regulation post infection. Similar to the earlier results, it was observed that a block in the PI3Kinase led to decrease in the Mcl-1 levels during infection. The infected cells were moreover readily susceptible to apoptosis with staurosporine after treatment with the inhibitor (Figure 22).

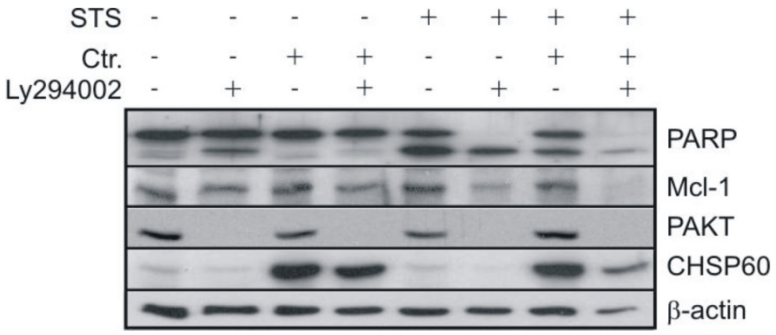


Fig. 22: Inhibition of PI3K sensitizes infected cells to apoptosis, by blocking the up-regulation of Mcl-1. END1 cells were treated with LY294002, 1 hour post infection with *Chlamydia*. Apoptosis was induced with Staurosporine and immunoblotting done to measure the levels of apoptosis by PARP cleavage, and the levels of Mcl-1 after the treatment with the inhibitor. pAKT was probed to

check for the inhibitor function, while chlamydial HSP60 levels were probed as an infection marker. As before, Bacterial Hsp60 (CHSP60) was used as an infection marker. END1 cells infected with *Chlamydia* had depleted Mcl-1 levels and were prone to apoptosis induced by staurosporine after treatment with a PI3K inhibitor. which.

2.5.5 Mcl-1 is required for the block in mitochondrial outer membrane permeabilization in the infected cells, upon apoptosis induction

Silencing of Mcl-1 led to the release of SMAC/DIABLO from the mitochondria upon apoptosis induction with TNF-α/CHX. Control and shMcl-1 cells were infected and treated with TNF-α/CHX. The cells were stained for SMAC and its release from mitochondria observed in the form of decrease in the punctuated staining in the cytosol. SMAC gets degraded in the cytosol upon release from the mitochondria, and this can be seen as a decrease in the SMAC release (Figure 23and 24).

The results were quantified by manually counting the number of cells with SMAC release and a graph plotted (Figure 25) It was calculated that the release of SMAC in the shMcl-1 cells was statistically significant with $P \leq 0.003$.

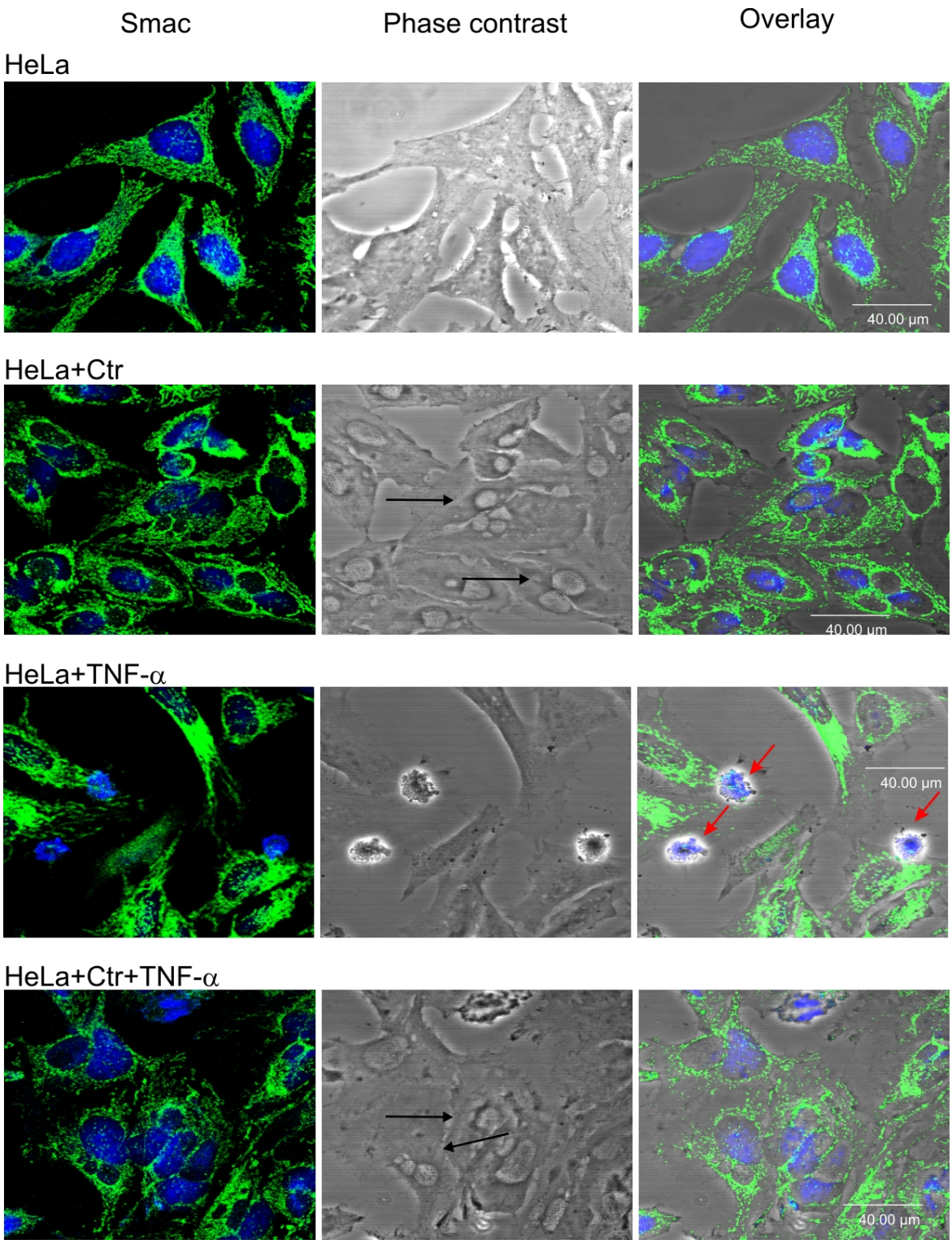


Fig. 23: A: Release of Smac from mitochondria upon apoptosis is blocked in infected cells. HeLa cells infected with *Chlamydia* (Ctr) and treated with TNF-α/CHX (TNF). Immunofluorescence analysis was done with Smac stained in green and nuclei stained blue. In healthy cells, Smac has a mitochondrial localisation, while in the apoptotic cells, Smac gets released into the cytosol where it gets degraded. Thus the loss of green staining reveals cells with mitochondrial permeabilization.

The red arrows mark such cells. The inclusions in the infected cells have been marked with black arrows.

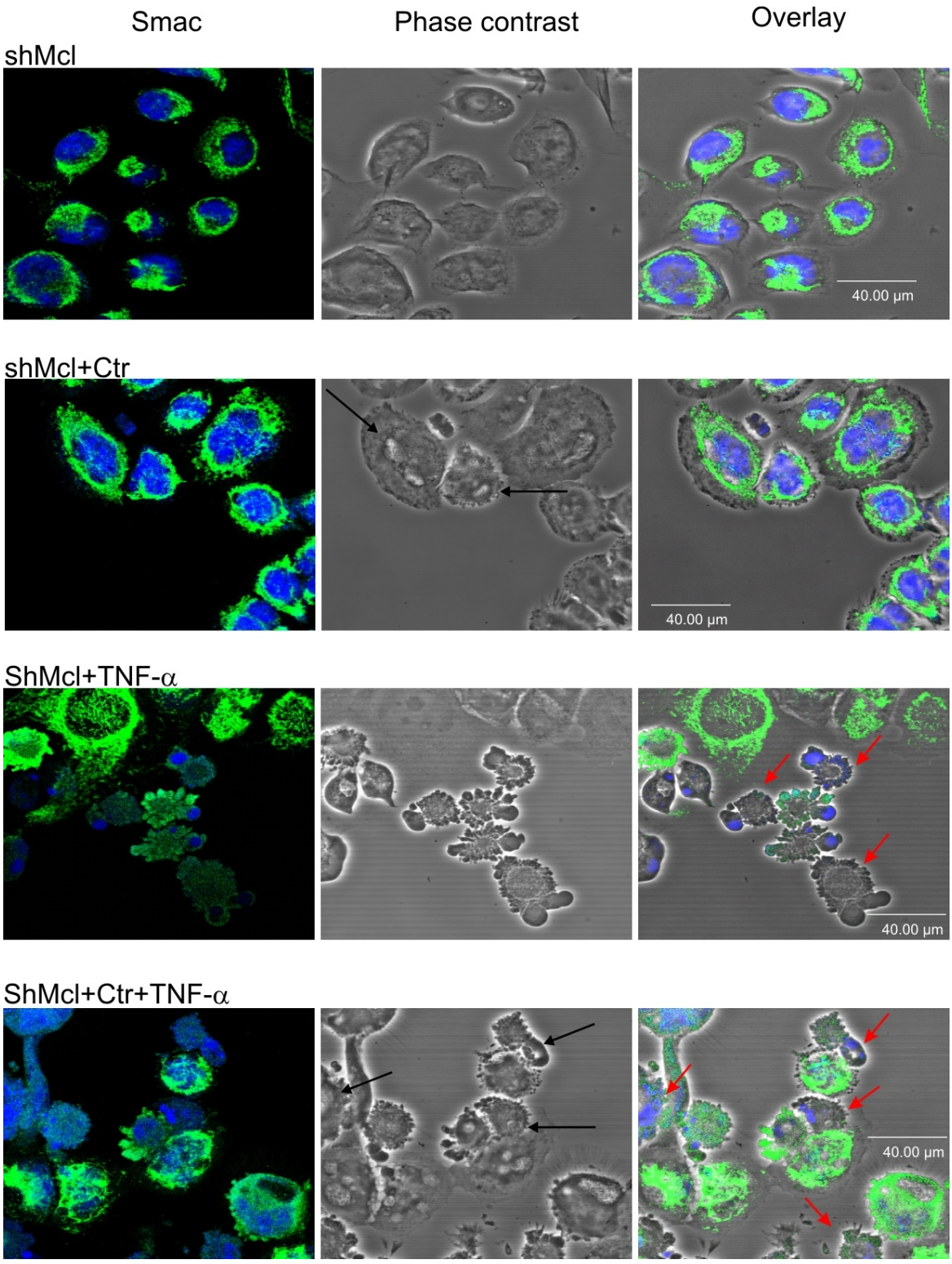


Fig. 24: Release of Smac from mitochondria upon apoptosis is rescued in infected cells in absence of Mcl-1. shMCl-1 cells infected with *Chlamydia* (Ctr) and treated with TNF-α/CHX. Immunofluorescence analysis was done with Smac stained in green and nuclei stained blue as

before. The red arrows mark such cells. The inclusions in the infected cells have been marked with black arrows.

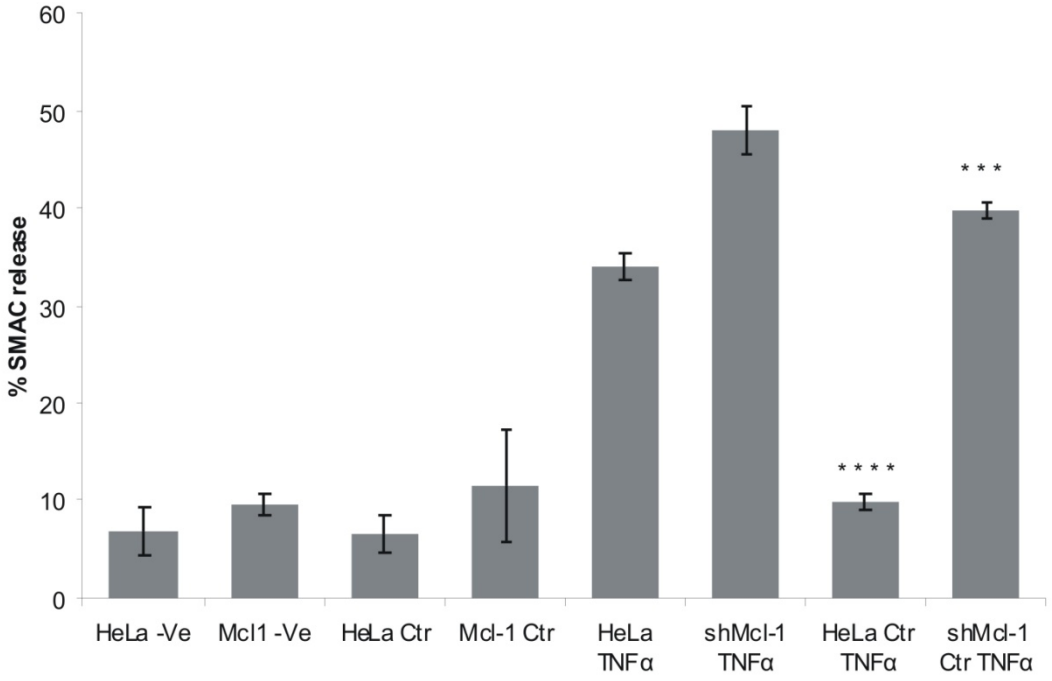
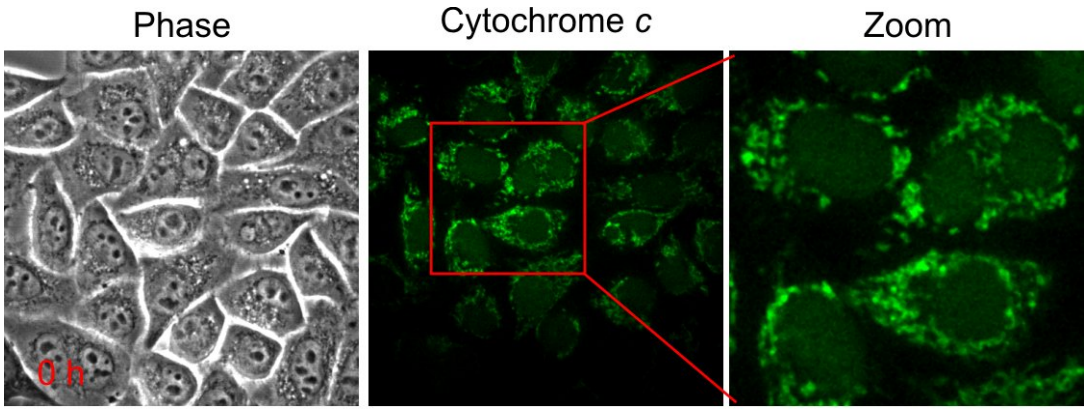


Fig. 25: Quantification of Smac release in the experiment from Figure 24 The total numbers of cells, and cells with loss of Smac staining were counted manually and the percetange calculated. – VE refers to uninfected cells. It was observed that there is an increase from 10 % to 40 % in the number of infected cells with Smac release upon apoptosis induction, after the knock down of Mcl-1.

2.5.6 Loss of Mcl-1 can rescue release of cytochrome *c* in the infected cells upon apoptosis induction

During the course of apoptosis induction in a cell by the mitochondrial pathway, cytochrome *c* gets released into the cytosol irreversibly commiting the cell to death. It has been shown that cell death occurs immediately (within minutes) following the release of cytochrome *c*. As a result, it is difficult to observe cytochrome *c* in the cytosol by immunostaining, because of the short time period between the cytochrome *c* release and the subsequent apoptosis of the cell. Moreover, the time point of release of cytochrome *c* differs slightly for the cells in a population, and therefore at any chosen time point (when the cells are fixed for microscopy) there would be only a few cells with cytochrome *c* localised in the cytosol, with a majority of cells either already rounded up or intact with no cytochrome *c* release.

Therefore, to monitor the release of cytochrome *c,* a HeLa cell line expressing a GFP-Cytochrome *c* construct was used and time lapse microscopy done. Infected and non-infected cells were treated with TNF-α/CHX and time lapse confocal microscopy used for detection of cytochrome *c*. The PI3K inhibitor LY294002 was used to obtain lower levels of Mcl-1 in the infected cells. As expected, it was seen that *Chlamydia* infected cells had no release of cytochrome *c* upon treatment with TNF-α/CHX. However, in the presence of LY294002, and subsequent reduction in the Mcl-1 levels, the release of cytochrome *c* occurred as normal. Once again it was observed that only the cells carrying a small inclusion were sensitized to apoptosis. The cells carrying larger inclusions had no release of cytochrome *c*.



Uninfected Control cells

Fig. 26: Cytochrome *c* is localized in the mitochondria of resting cells. Image from time lapse microscopy of untreated HeLa-GFP-Cyt*c* cells.

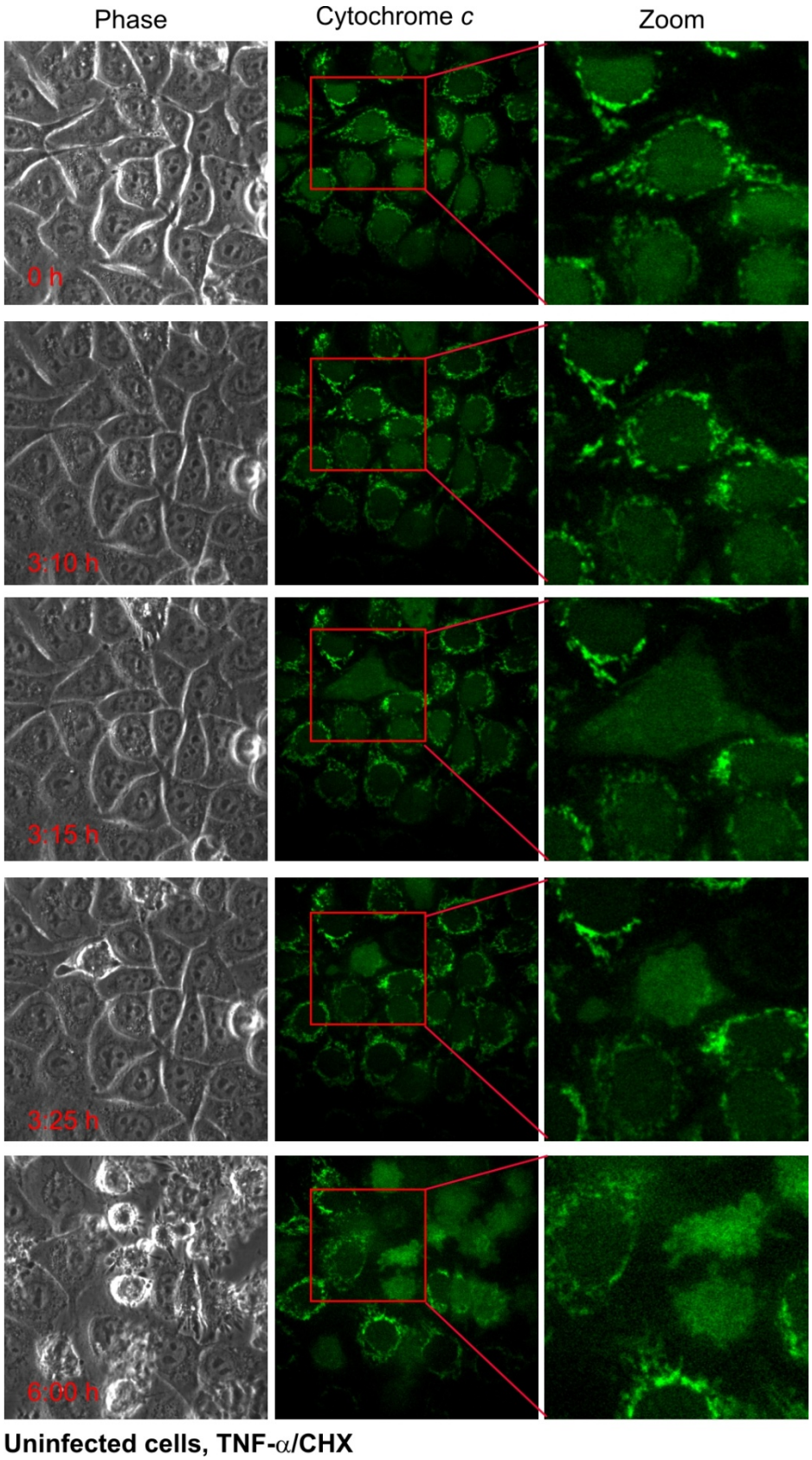


Fig. 27: Cytochrome *c* is released from the mitochondria on apoptosis induction. Images from time lapse microscopy of HeLa-GFP-Cyt*c* cells treated with TNF-α/CHX. The time in hours after TNF-

α/CHX treatment is marked in red. At the 3:15 h time point the cell inside the marked field shows a release of cytochrome *c*. At the next time point, the cell is seen to be rounded up with advanced apoptosis. 6 h post TNF-α/CHX treatment, most cells have undergone cytochrome *c* release and subsequent apoptosis.

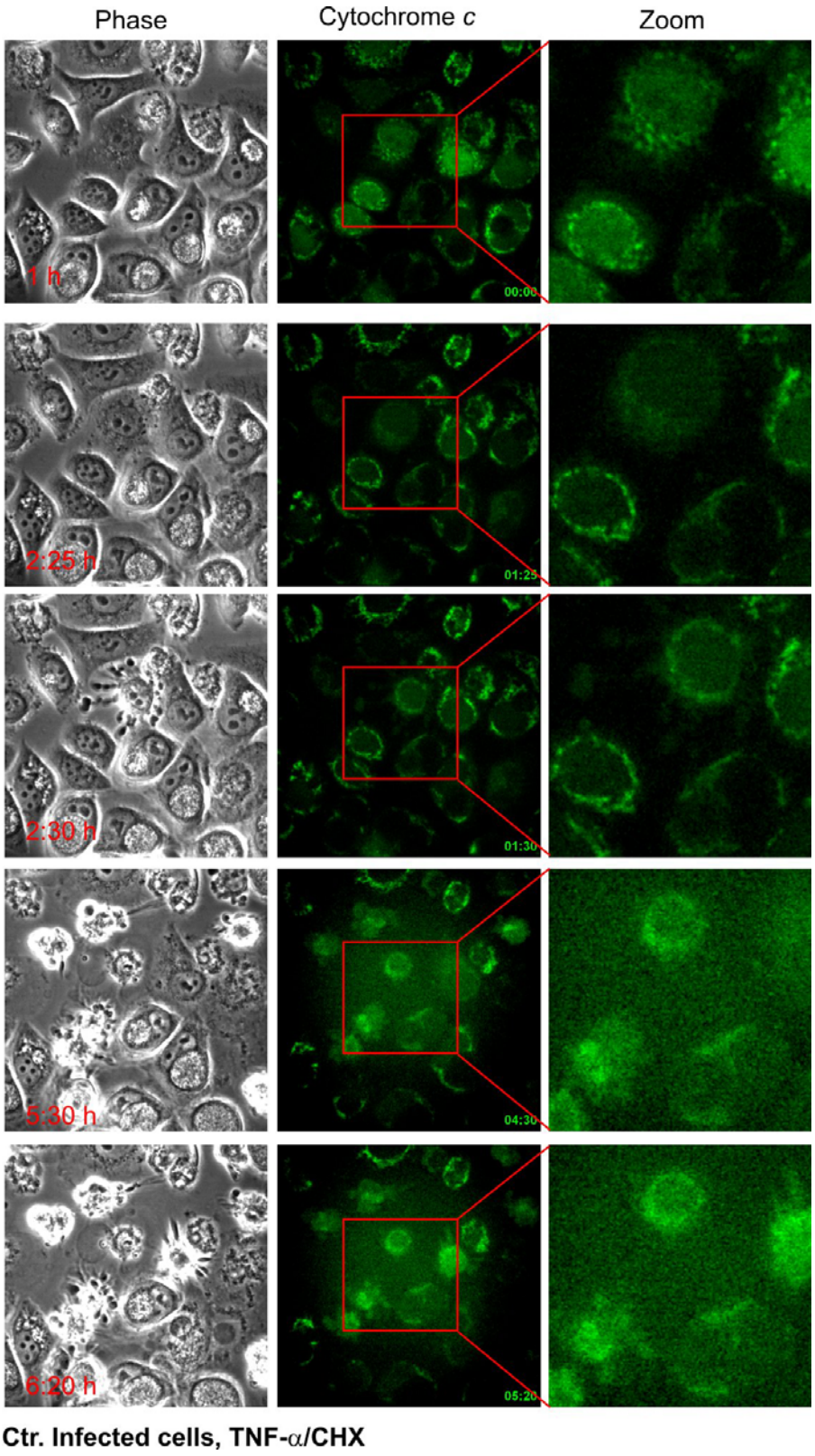


Fig. 28: HeLa cells infected with Ctr. And treated with TNF-α/CHX

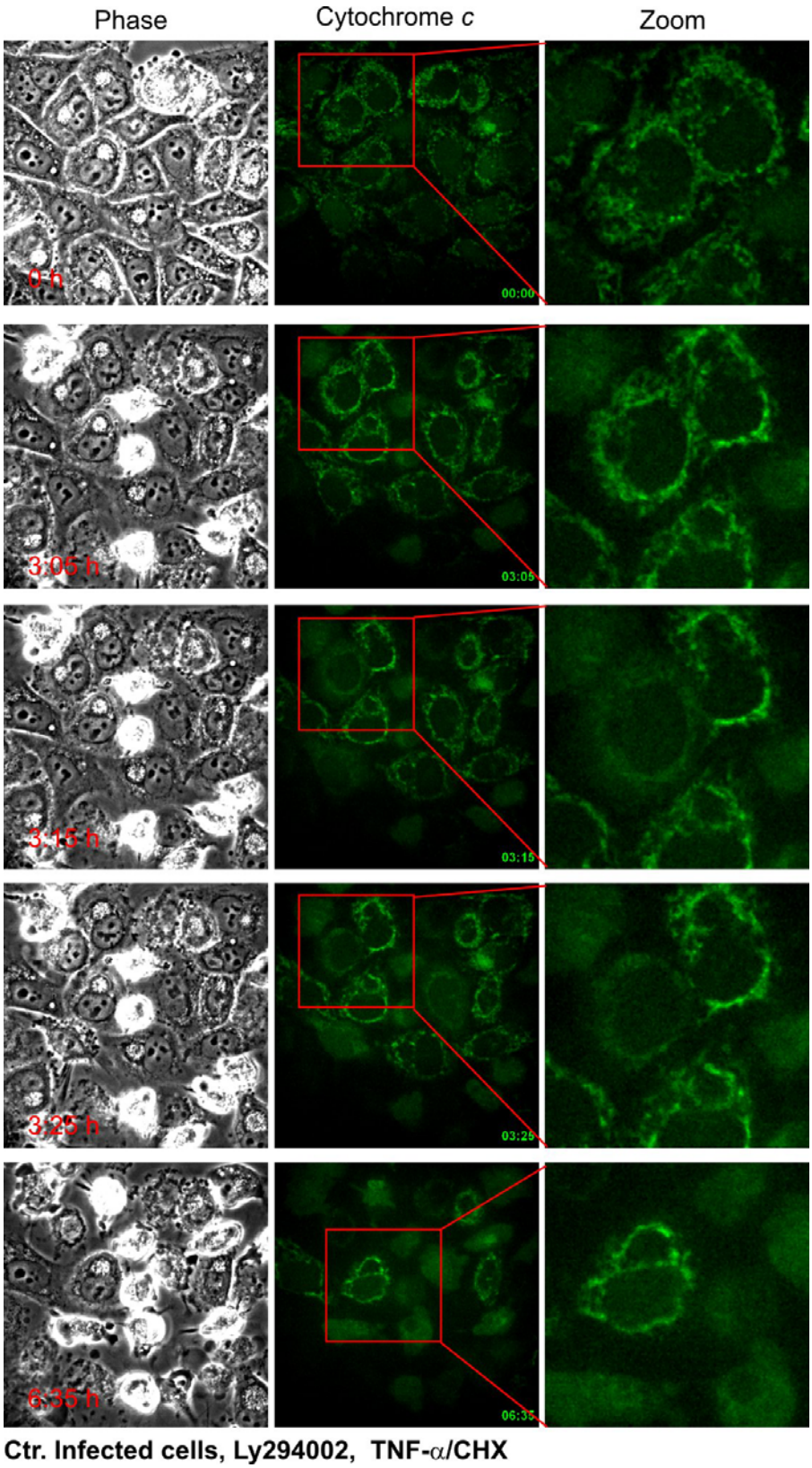


Fig. 29: HeLa cells infected with Ctr. And treated with LY294002 and TNF-α/CHX

2.5.7 Loss of Mcl-1 leads to caspase-9 activation in the infected cells, upon apoptosis induction

Mcl-1 knock down and control cells were treated with TNF-αc and Staurosporine with and without infection. Processing of caspase-9 and the extent of apoptosis induction was determined by immunoblotting. Caspase-9 cleavage was abrogated in the infected control cells. However, in the shMcl-1 cell lines, cleaved caspase-9 fragments were observed. It should be noted that although cleavage of caspase-9 is not a marker of its activation, the cleavage is a probable intermediatary step of apoptosis, which is absent in the *Chlamydia* infected cells. Loss of Mcl-1 leads to the cleavage of caspase-9 and the subsequent cell death by apoptosis, as judged by the PARP cleavage in the Figure 30

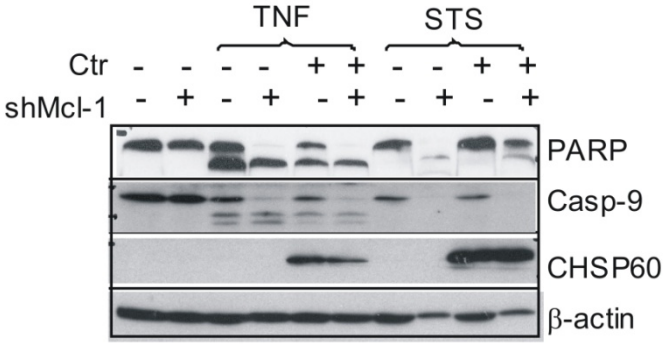


Fig. 30: Caspase-9 processing and subsequent apoptosis occurs in infected cells in absence of Mcl-1. Control and shMcl-1 cells were infected and treated with TNF-α/CHX or Staurosporine (STS). The samples were probed for PARP and caspase-9 levels by immunoblotting. In the Staurosporine treated samples, cleavage of caspase-9 is detected by the loss of the caspase-9 proform. In the control cells, caspase-9 processing was blocked, but in the absence of Mcl-1 the caspase-9 processing occurred as normal upon apoptosis induction by TNF-α/CHX or staurosporine.

Thus in the absence of Mcl-1 there is mitochondrial permeabilization followed by Smac and cytochrome *c* release and the consequent caspase-9 and caspase-3 activation. This indicates that Mcl-1 is the most important factor constituting the block in the apoptosis upstream of the mitochondria in the *Chlamydia* infected cells.

2.6 *HIF-1α gets stabilized and modulates the expression of anti-apoptotic proteins during* C. trachomatis *infection*

As described before, HIF-1 had been identified as a regulator involved in the different pathways likely to be involved in apoptosis resistance in the infected cells. We therefore analyzed the state of Hypoxia Inducible Factor-1 (HIF-1) during infections. As described in the introduction, HIF-1 is composed of HIF-1β and HIF-1α subunits. While HIF-1β is constitutively expressed in cells, HIF-1α is regulated by the oxygen levels in the cells. Under normoxic conditions, HIF-1α gets degraded in the cytoplasm of the cells, whereas in hypoxic conditions it gets stabilized and accumulates in the cell. As such HIF-1α is present at very low levels in healthy cells.

2.6.1 HIF-1α gets stabilized at the protein level during early infection, but gets degraded during late time points of infection

We checked for the levels of HIF-1α during infection with *C.trachomatis*. At first it was seen that 24 hours post infection, there was no increase in the levels of HIF-1α. However, a time course of infection revealed that HIF-1α is first up regulated and then degraded during the infection.

As seen in Figure 31, the levels of HIF-1α are increased around 12 hours of infection. The stabilization required active growth of the bacteria, as there was no increase in HIF-1α levels when cells were treated with heat inactivated *Chlamydia*. $CoCl_2$ was used to simulate hypoxia in cells leading to detectable levels of HIF-1α, as a positive control.

The lower figure shows the degradation of HIF-1α 24 hours post infection. Interestingly, treatment of the infected cells with the MEK-1 inhibitor UO126 blocked the degradation, suggesting a role of the ERK pathway in infection induced modulation of HIF-1α - either the bacterial growth is affected in such a way as to block hypoxic conditions in the cells, or the degradation of HIF-1α itself requires the ERK pathway.

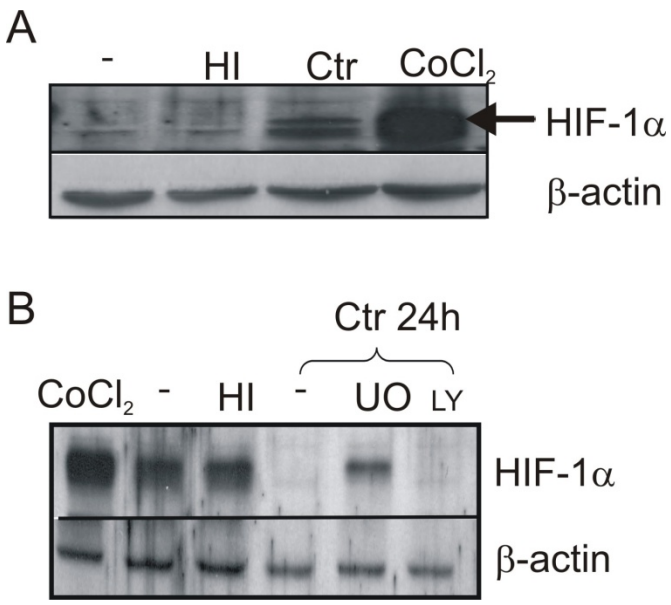


Fig. 31: HIF-1α expression is up-regulated in cells during early time points of Ctr. Infection. **A:** HeLa cells were infected with heat inactivated *Chlamydia* (HI) or *Chlamydia* and lysed after 12h. The samples were probed for the levels of HIF-1α by immunoblotting. The last lane shows a positive control of cells treated with $CoCl_2$ for 4 h. $CoCl_2$ simulates hypoxia in the cells leading to a strong up-regulation of HIF-1α.

B: Cells were infected with (active and heat inactivated) Chlamydia for 24 h and probed for Hif-1α as before. Cells were infected with or without the MAPK inhibitors UO126 (10 μM) and LY294002 (60 μM). HI referes to Heat Inactivated Chlamydia. HIF-1α was strongly degraded 24 h post infection, but this degradation was blocked in the presence of U0126.

2.6.2 HIF1-α is not transcriptionally regulated during infection

The accumulation of HIF-1α is not due to increased expression at mRNA level, but due to stabilization at the protein level. When an infection time course was performed it was seen that the mRNA levels of HIF-1α remained more or less constant through the course of the infection. In contrast, Mcl-1 was up regulated as described before.

This confirms that the accumulation of HIF-1α in the cells is due to stabilization in the cytosol, possibly due to hypoxic conditions generated by the presence of bacteria in the cell.

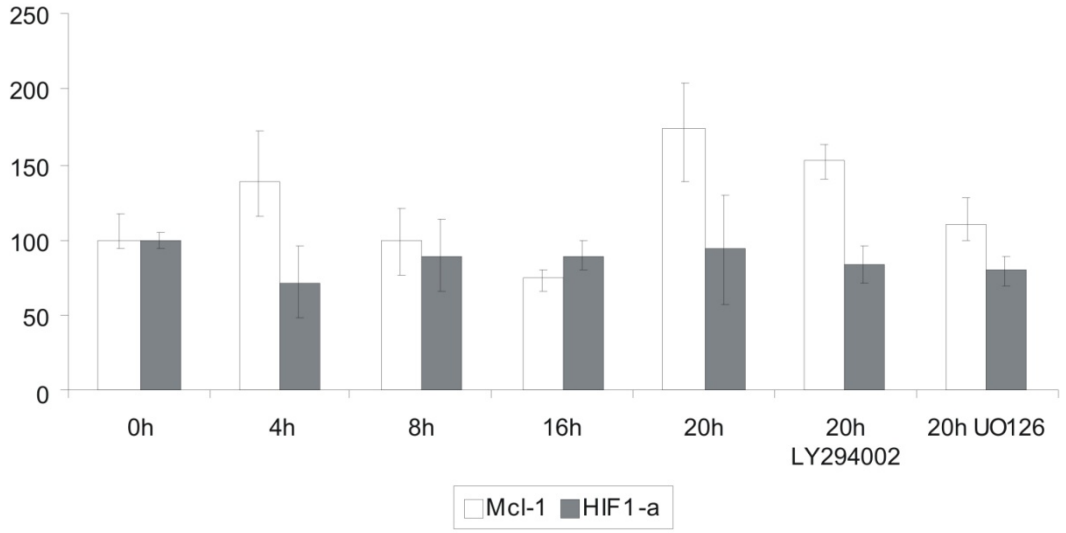


Fig. 32: HIF-1α is not transcriptionally regulated during *chlamydial* infection. HeLa cells were infected for the indicated time points with or without the presence of MAPK inhibitors – 10μM UO126 or 60μM LY294002. The expression of Mcl-1 and HIF-1α was analysed by q-RT PCR. As stated before, Mcl-1 gets up-regulated 20 hours post infection and inhibition of MEK-1 with LY294002 reverses this effect. On the other hand, HIF-1α is not regulated by the infection at the mRNA level.

2.6.3 HIF-1 α gets translocated to the nucleus during infection

For achieving the proper functionality of the Hypoxia Inducible Factor, HIF-1α needs to be translocated to the nucleus, where it binds to HIF-1β to form the transcriptionally active HIF-1 complex. We therefore carried out immunofluorescence studies to look for the localisation of HIF-1α during the infection time course. In the absence of infection, it was seen that HIF-1α was localized to the cytosol. However after 12 hours of infection, HIF-1α expression levels were slightly increased and a nuclear localisation was also seen. As seen before in the immunoblot studies, HIF-1α was seen to get degraded around 24 hours of infection.

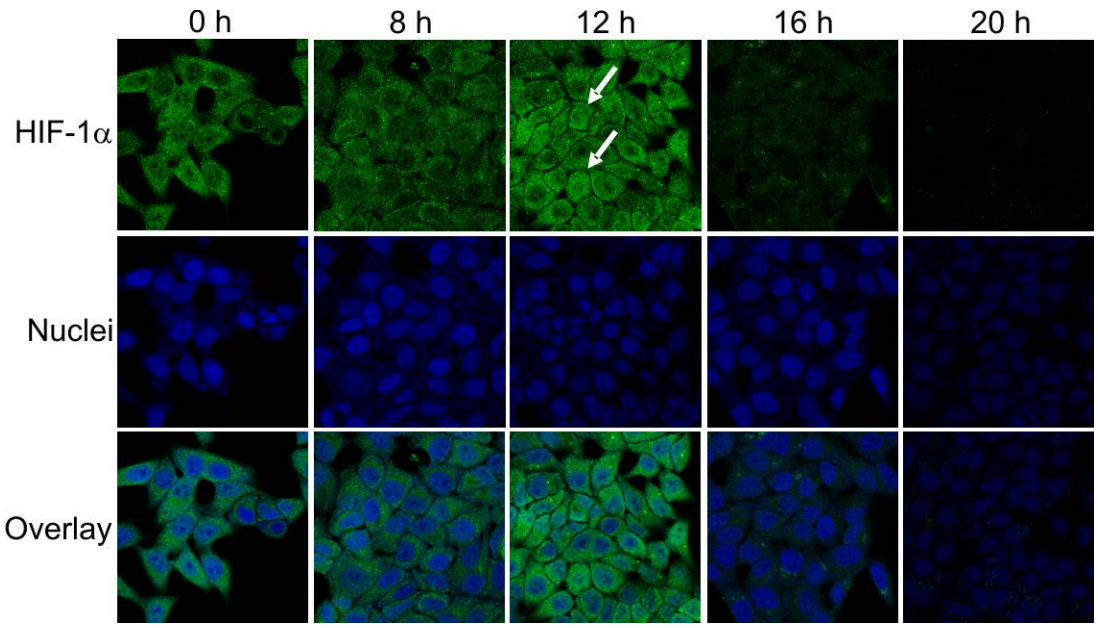


Fig. 33: HIF-1α translocates to the nucleus 12h post Ctr. Infection. HeLa cells were infected with *Chlamydia* and fixed at the different indicated time points. The cells were stained for Hif-1α in green (upper row), and the nuclei were stained blue with hoescht. Hif-1α has a cytoplasmic localization in healthy cells. 12 hours post infection, cells with Hif-1α translocated to the nucleus can be observed (marked with arrows). After 16h of infection, there is a marked decrease in the Hif-1α levels in the cells.

2.6.4 Hypoxia Inducible Factor is required for the transcriptional up-regulation of Mcl-1 after infection with *C.trachomatis*

HIF-1 has been shown to be a transcription factor responsible for regulating Mcl-1 [112]. We therefore checked for the mRNA levels of Mcl-1 in the absence of HIF-1α after chlamydial infection. In the control cells transfected with an siRNA against Luciferase (siLuci) Mcl-1 was up regulated in response to the infection as observed before. However, after HIF-1α knock down, the mRNA levels of Mcl-1 did not increase like in the control cells. The result was statistically significant, with a p-value ≤ 0.01. This proved that Mcl-1 is transcriptionally up regulated through HIF-1 during the infection (Figure 34).

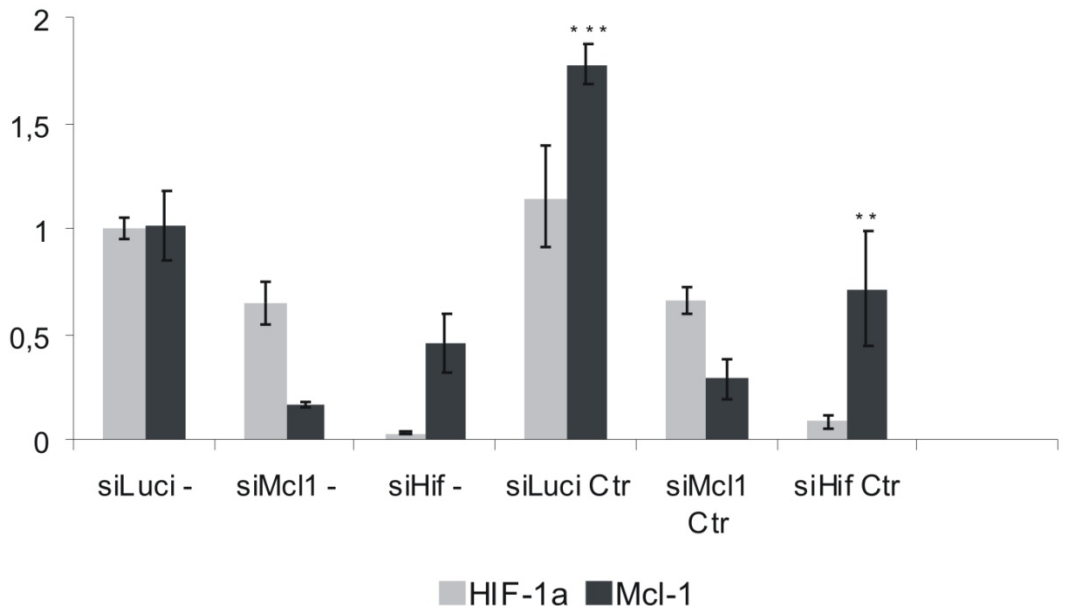


Fig. 34: Mcl-1 up-regulation is blocked at the mRNA level in absence of HIF-1α, in the Ctr. infected cells. HeLa cells were transfected with siRNA against Luci, Hif-1α or Mcl-1 and infected with *Chlamydia* for 24 hours. The expression levels of Hif-1α and Mcl-1 mRNA were analyzed by q PCR. "–" refers to uninfected samples. Mcl-1 levels were increased as expected, in the control (siLuci) after chlamydial infection. However, after knock down of Hif-1α, the chlamydial infection failed to up-regulate the levels of Mcl-1.

2.6.5 Loss of HIF-1α sensitizes *Chlamydia* infected cells to apoptosis

siRNA against HIF-1α, Mcl-1 and Allstar were used to transfect cells, and then the cells were infected and treated with TNF-α/CHX as before (Figure 35). Knock down of Mcl-1 had a strong sensitizing effect on the infected cells. However, knock down of HIF-1α had a weak sensitizing effect as seen in the PARP cleavage in the figure. The phase contrast pictures of the apoptotic cells also show the same observation. The low sensitizing effect might be due to the low levels of knock down achievable.

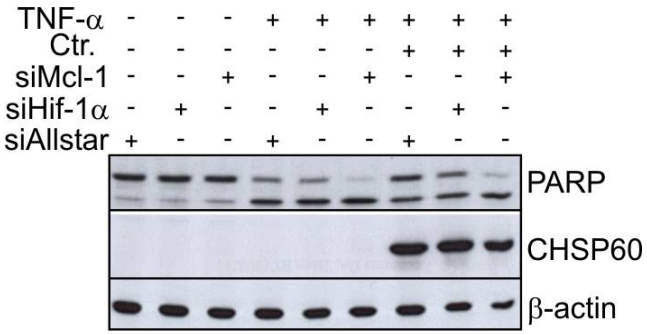


Fig. 35: Loss of HIF-1α sensitizes *Chlamydia* infected cells to apoptosis. Cells were transfected with siRNAs against Allstar, Hif-1α or Mcl-1 and infected with *Chlamydia* after 48 hours. Apotosis was induced with TNF-α/CHX after 24 h post infection and the PARP cleavage measured by immunoblotting. Bacterial Hsp60 (CHSP60) was used as an apoptosis marker. HeLa cells transfected with siHIF-1α or siMcl-1 were sensitized to apoptosis with TNF-α/CHX after infection.

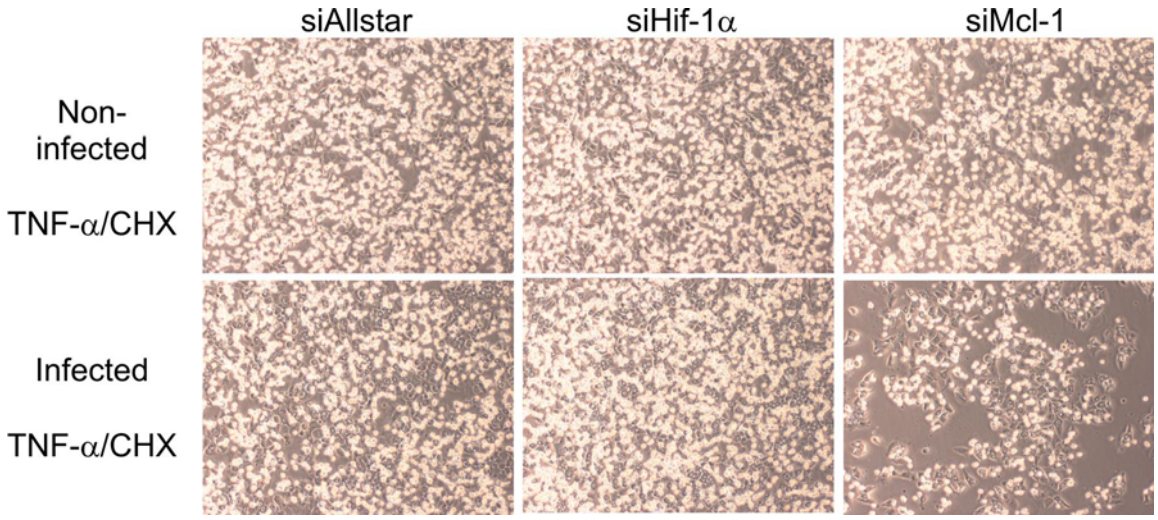


Fig. 36: Phase contrast images of cells from the experiment in Figure 35. The knock down of Hif-1α was seen to have a noticeable sensitization of the infected cells to apoptosis, as compared to the control cells.

Table 1 **List of high confidence hits identified by RNAi screen which sensitize infected cells for TNF-α/CHX induced apoptosis.**

	Gene Symbol	Name	Comments; Known functions
	RAS/MAPK family		
1	CRAF	v-raf-1 murine leukemia viral oncogene homolog 1	Proliferation, differentiation, anti-apoptosis
2	AKT1	v-akt murine thymoma viral oncogene homolog 1	Anti-apoptotic, cell growth
3	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	RAS family member, induces cytoskeletal changes
4	PRKCG	protein kinase C, gamma	PKC family member, various functions
5	RAB11B	RAB11B, member RAS oncogene family	Vesicular trafficking, cytokinesis
6	MAPK14	mitogen-activated protein kinase 14	Stress activated protein kinase
7	**NLR family**		
8	NLRP14	NLR family, pyrin domain containing 14	Inflammatory response; innate immunity
9	NLRP5	NLR family, pyrin domain containing 5	Inflammatory response, innate immunity
10	NLRP6	NLR family, pyrin domain containing 6	Inflammatory response; innate immunity
	Mitochondrial proteins		
11	VDAC2	voltage-dependent anion channel 2	Role in cell death; inhibitor of BAK
12	MCl-1	myeloid cell leukemia sequence 1	Anti-apoptotic Bcl-2 family member
13	ATP5A1	ATP synthase, H+ transporting, mitochondrial F1	ATP generation;

		complex, alpha subunit 1, cardiac muscle	metabolism
	Receptor-associated proteins		
14	MET	met proto-oncogene (hepatocyte growth factor receptor)	Tyrosine Kinase family oncogene
15	TRAF6	TNF receptor-associated factor 6	Adaptor protein – involved in NF-kappaB activation
16	**Cytoskeletal factors**		
17	NCK1	NCK adaptor protein 1	Actin nucleation and assembly
18	RDX	radixin	Links cytoskeleton and plasma membrane
19	KIF5B	kinesin family member 5B	Microtubule based motor protein
20	KRIT1	KRIT1, ankyrin repeat containing	Plays role in integrin mediated adhesion/migration
	Nuclear factors		
21	MEFV	Mediterranean fever	Inflammatory response
22	RBBP4	retinoblastoma binding protein 4	Modulates E2Fs
23	EIF5A	eukaryotic translation initiation factor 5A	Translation initiation and cell viability
24	NAP1L1	nucleosome assembly protein 1-like 1	mitotic regulation
25	SKP1A	S-phase kinase-associated protein 1A (p19A)	Cell cycle regulation
	Other hits		
26	BIRC3	baculoviral IAP repeat-containing 3	Caspase inhibitor ; anti apoptotic; Positive control

27	BIRC4	baculoviral IAP repeat-containing 3	Positive control, major caspase inhibitor, anti apoptotic
28	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Amino acid transport; Calcium homeostasis
29	CLTA	clathrin, light chain (Lca)	Structural component of endocytic vesicles
30	PAGE5	P antigen family, member 5 (prostate associated)	Not known
31	IK	IK cytokine, down-regulator of HLA II	Immune response
32	PABPC4	poly(A) binding protein, cytoplasmic 4 (inducible form)	stability of labile mRNA
33	NOS1	nitric oxide synthase 1 (neuronal)	NO generation
34	NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)	NO generation

3 Discussion

Inhibition of apoptosis constitutes an important immune escape mechanism for certain bacteria, helping them to stay inside the host cell for longer periods of time. *Chlamydia* spp. can robustly block the mitochondrial pathway of apoptosis, thus protecting infected host cells against various stimuli.

In the present study, it was seen that the chlamydial infection could block the apoptotic pathway at multiple levels by modulation of specific host cell factors. Most prominent among these were the anti-apoptotic proteins Mcl-1 and cIAP-2 which were found to be up regulated during the chlamydial infection, and were seen to be absolutely required for apoptosis inhibition.

Although cIAP-2 was the only member of the IAP family that was up regulated, it was seen that the presence of cIAP-1 and XIAP was also needed to block apoptosis in the infected cell. That these IAPs work together in a concerted mechanism was further confirmed when it was observed that cIAP-1, cIAP-2 and XIAP exist in large native heteromeric complexes in a cell. When XIAP expression was reduced by RNAi, the presence of other members in the large complex was not detected by the gel filtration technique employed.

Previous reports have shown that the MAPK pathways get activated early during the chlamydial infections [164]. Consistent with these reports, Ras-GTP could be pulled down and ERK and AKT phosphorylation could be observed after the infection. It was observed that not only were the MAPK pathways activated during the infection, but these were also required for the apoptosis inhibition. A high throughput RNAi screen to identify host factors required for the apoptosis resistance, confirmed that the MAPK pathways were critically involved[2]. Six factors associated with the RAS signaling machinery were found to be required for conferring apoptosis resistance. Other factors identified included Mcl-1, the tyrosine receptor kinase (c-Met) as well as some mitochondrial, nuclear and cytoskeletal proteins.

Analysis of the MAPK pathways revealed that these pathways confer apoptosis resistance in the infected cells, by regulating the expression of both Mcl-1 and cIAP-2. The infection led to an increase in expression of both Mcl-1 and cIAP-2, which was reversed in the

[2] This work was performed by Machuy et. al.

presence of the MEK-1 inhibitor UO126 or the PI3K inhibitor LY294002. The Raf/MEK/ERK pathway was required for the transcriptional up-regulation of Mcl-1. The PI3K/AKT pathway was important for the stabilization of the Mcl-1 and cIAP-2 protein level.

Detailed pathway analysis of the factors identified from the screen revealed HIF-1α as a key player involved in the differential expression of the survival genes during infection. Consistent with this, it was found that HIF-1α does get stabilized and accumulates in the cell during an early time point of infection. This fits well with earlier reports showing that the p44/42 MAPK is critically required for the increased expression of HIF-1α induced by bacterial lipopolysaccharide [165]. Rupp *et. al.* have shown that *Chlamydia pneumoniae* regulates the HIF-1α stabilization in human host cells [124]. Here we observed that *Chlamydia trachomatis* infection similarly regulates HIF-1α levels in the cell, and moreover, this plays a key role in apoptosis inhibition.

As shown previously, the transcription factor, HIF-1, regulates the expression of various survival genes, including cIAP-2 and Mcl-1 [112,116]. Consistent with this, both Mcl-1 and cIAP-2 are transcriptionally up-regulated after the chlamydial infection. The involvement of HIF-1 in the chlamydial induced apoptosis resistance was confirmed by silencing of HIF-1α that blocked the over-expression of Mcl-1 at the mRNA level, in the infected cells.

3.1 Requirement of bacterial and host cell protein synthesis for apoptosis inhibition

The ability of *Chlamydia* spp. to block host cell apoptosis has long been known. Early reports suggested that chlamydial protein synthesis is essential for the resistance since treating the cells with the prokaryotic transcription inhibitor rifampin or the translation inhibitor chloramphenicol abrogated apoptosis inhibition. It was also reported that since *Chlamydia* could block apoptosis even in the presence of the eukaryotic translation inhibitor cycloheximide, host cell synthesis was not required for apoptosis inhibition [148]. However, it was observed that a block in up-regulation of host proteins like Mcl-1 or IAPs could sensitize cells to apoptosis, which shows that host protein production is required by *Chlamydia* for imparting resistance. In fact, around 32 host cell factors were identified that were involved in the apoptosis resistance, with the help of an RNAi screen. This

discrepancy about the involvement of host cell protein synthesis could be because of the inability of cycloheximide in inhibiting translation completely. In fact even during the presence of high concentration of cycloheximide, proteins, among others (Mcl-1), were observed to be overexpressed during infection.[1]

3.2 Block of mitochondrial outer membrane permeabilization during infection

It has been shown that the chlamydial infected cells failed to achieve the activation of Bax and Bak, and consequently mitochondrial permeabilization is not achieved [149,150]. Reports have also shown that infection fails to impart resistance in type 1 cells, which do not require the mitochondrial pathway for activation of the effector caspases [151]. It becomes clear that *Chlamydia* confers a block at the mitochondrial level which explains the resistance to apoptosis initiated through different pathways that collude at the mitochondria.

3.2.1 BH3 only proteins are not degraded during *Chlamydia trachomatis* infection

Previous reports attributed the block in mitochondrial permeabilization during the chlamydial infection to a broad scale degradation of the different BH3-only proteins in the cell [150,152,153,154]. The Chlamydial protease-like activity factor (CPAF) was shown to be responsible for targeting those active proteins with an exposed BH3 domain, for degradation, implying that *Chlamydia* destroy most, if not all, active BH3-only proteins. In absence of the BH3-only proteins, death signals cannot be transmitted to the mitochondria, and this could account for the block in apoptosis upstream of the mitochondria. To verify this, the levels of the different BH3 only proteins were extensively analyzed in an infection time course experiment. Immunoblot as well as immunofluorescence studies with validated antisera showed that there was no major change in the levels of these proteins after infection. To rule out differences in infection conditions, the activity of CPAF during the infection was checked by measuring the levels of its substrate - Keratin 8. CPAF has been reported to be a protease secreted by *Chlamydia* that is both necessary and sufficient to cleave Keratin 8 to modify the host cell cytoskeleton [166]. It is probable that the

[1] This work was performed by Rajalingam et. al. in the Rudel group.

contradictory results observed here could be because of differences in experimental conditions including different cell types, *Chlamydia* serovars etc.

In an elegant experiment to analyze the extent of apoptosis inhibition in the infected cells, it was noticed that the cytosolic extract from chlamydial infected cells resisted the activation of caspase-3 even upon treatment with cytochrome *c* [155]. This suggests a block in the apoptotic pathway downstream of the mitochondria. The BH3 only proteins can regulate the mitochondrial outer membrane permeabilization. Degradation of the BH3 only proteins, would not account for this block in caspase-3 activation after cytochrome *c* treatment. It becomes clear that the degradation of the BH3-only proteins is not the only mechanism employed by *Chlamydia* to achieve the strong and widespread inhibition to apoptosis induced by various stimuli.

3.2.2 Up-regulation of Mcl-1 is responsible for inhibiting mitochondrial outer membrane permeabilization during *C. trachomatis* infection

In the present study, the over-expression of Mcl-1 during infection was seen to cause the block in the apoptotic pathway upstream of the mitochondria. There was a time dependent increase in the mRNA and protein levels of Mcl-1. In the absence of Mcl-1, the infected cells showed mitochondrial outer membrane permeabilization, Smac and cytochrome *c* release, caspase-9 and caspase-3 activation and finally cell death upon treatment with apoptosis inducers. Silencing of Mcl-1 with RNAi or its down-regulation by inhibiting the MAPK pathways sensitized infected cells to apoptosis.

Smac gets degraded in the cytosol upon release from the mitochondria during apoptosis [167]. As such, Smac release from the mitochondria was detected by its loss of staining in immunofluorescence studies. However, detecting the release of cytochrome *c* was more difficult, because of the small window of time between its release into the cytosol, and the rounding up of cell for apoptosis. Once a cell “rounds up” due to apoptosis, it is difficult to see in the immunofluorescence images, if the cytochrome *c* is localized in the mitochondria or the cytoplasm. Therefore, time-lapse microscopy was employed using a HeLa cell line expressing GFP-cytochrome *c and thus the* the translocation of cytochrome *c* to the cytosol was detected.

To check for the role of Mcl-1 in the release of cytochrome *c* in the infected cells, the PI3K inhibitor, LY294002, was employed to deplete Mcl-1 levels. As observed before, blocking PI3K/AKT pathway with, LY294002 could inhibit the up-regulation of Mcl-1 in the infected cells. Time lapse microscopy clearly showed the release of cytochrome *c* in the

infected cells, in the presence of the inhibitor, confirming the role of PI3K/AKT pathway in blocking the mitochondrial membrane permeabilization through Mcl-1.

Recently, the role of Mcl-1 in sustaining bacterial infection has also been shown for *Helicobacter pylori* infections in the gut epithelia. It was observed that *H.pylori* infection could suppress the apoptosis initiated by the host defence mechanisms in the infected cells. The infected cells had increased levels of activated-ERK and Mcl-1 which were required for apoptosis inhibition [168]. Similarly, Salmonella enterica serovar Typhimurium inhibits camptothecin-induced apoptosis in HeLa and rat small intestine epithelial cells by activating the PI3K/Akt pathway [169]. Anaplasma phagocytophilum prevents apoptosis in neutrophils by activation of p38 MAPK [170].

It appears that the activation of MAPK pathways for the up-regulation of Mcl-1 could be a more common strategy for resisting host apoptotic pathways during bacterial infection.

3.3 Block in caspase-3 activation during infection

Chlamydia also block the apoptotic pathway downstream of the mitochondria, at the caspase-3 level. Analysis of cleaved caspase-3 immunoblots from the infected cells showed that with the growth of the inclusion, there is a block in the autocatalytic conversion of the cleaved caspase-3 p19 fragment into the p17 fragment. This incomplete processing of caspase-3 is similar to that seen after apoptosis induction in cells with a Smac knock down [171]. The block in caspase-3 processing could therefore be because of a block in mitochondrial permeabilization and/or because of the IAPs which directly bind and inhibit caspase activation.

The Inhibitor of Apoptosis Proteins, IAPs, are important regulators of apoptosis that bind directly to caspase-3 and caspase-9 with their BIR domains, and thus block their activation. During the infection, cIAP-2 gets up regulated at the protein levels, while the levels of cIAP-1 and XIAP remained unaltered. The increase at protein level was detected after around 16 hours of infection which concurs with the time post infection of apoptosis resistance in the infected cell. Immunoblot analysis for cleaved caspase-3 showed that in the absence of cIAP-2, the block in caspase-3 processing was attenuated.

Recently, some reports have raised doubts over the ability of cIAP-2 and cIAP-1 in directly inhibiting the catalytic activity of caspases [172]. However, these data clearly show that in the presence of high levels of cIAP-2, there is incomplete activation of caspase-3 and this

effect is abrogated when cIAP-2 levels were reduced by RNAi. It should be noted that the role of increased expression of cIAP-2 in inhibiting apoptosis has since been confirmed elsewhere. When mouse macrophages were treated with LPS, there was an up-regulation of cIAP-2 which provided resistance to Fas induced apoptosis in the cell. Moreover, when the macrophages from cIAP-2 knock-out mice were treated with LPS, there was widespread apoptosis confirming the requirement of cIAP-2 in the apoptosis resistance [173].

3.3.1 IAPs exist in the form of native heteromeric complexes

The presence of cIAP-1 and XIAP was important together with the high levels of cIAP-2 to block apoptosis. Apparently, these IAPs might be working in a concerted manner, as part of a functional complex that blocks caspase activation. To check for this, gel filtration studies were done with the cytosolic extracts from HeLa cells. cIAP-1, cIAP-2 and XIAP were indeed detected in high molecular weight fractions of around 400 kDa. Survivin was not detected in the IAP-containing high molecular weight fractions.

The presence of IAPs in a complex has been shown before. Dohi et al. reported that XIAP and survivin form a heteromeric complex in response to cell death simulation *in vivo* [174]. Silke et *al*. observed a direct interaction of XIAP with overexpressed cIAP-1 via their RING domains [175]. In the present work, although XIAP, cIAP-1 and cIAP-2 interaction was detected, survivin was not observed to be associated with the IAP complex even during apoptosis induction. Interestingly, loss of survivin also did not influence the resistance to apoptosis in *Chlamydia* infected cells. In contrast, XIAP which was required for the apoptosis resistance was crucial for the stability of the IAP complex. In the absence of XIAP in the XIAP knock down cell lines, the complex seemed to break down- cIAP-1 and cIAP-2 were no longer found in the 400 kDa fraction, but mostly in the monomeric fraction. cIAP-2 was also detected in a 1 MDa fraction, suggesting its recruitment to a different complex under these conditions. Further immunoblot studies revealed that depletion of XIAP affects the stability of cIAP-2 in the infected cells – cIAP-2 levels are not increased in the absence of XIAP upon infection[1]. The presence of XIAP is thus vital for the recruitment of cIAP-1 and cIAP-2 to the 400 kDa complex which in turn might be important for the stability of these IAPs.

To check what happens to the complex during apoptosis, gel filtration studies were performed on HeLa and Jurkat cells induced to apoptosis. The complex was seen to get disrupted, and only a low amount of IAPs was detected in the fractions. Interestingly, cIAP-1 was detected in a different fraction, around 850 kDa in size, in the apoptotic cells. During apoptosis, Smac is known to bind to IAPs to inactivate them. To check for the interaction of Smac to the IAP-IAP complexes, the high molecular-weight fractions from the gel filtration were probed with immunoblotting. Smac was found to occur in all the high molecular weight fractions containing the IAPs, but not in the fractions containing the monomeric IAPs. Smac could thus be interacting with the functional IAP complex, inhibiting it during apoptosis. Further, we also detected caspase-3 together with Smac and the IAPs in the high molecular weight fractions from the apoptotic cells. These results suggest that the heteromeric IAP complex might be the functional IAP unit, rather than the individual monomers. However, further work is required to confirm this. It would be interesting to isolate the IAP complexes, and analyze their inhibitory effect on caspase-3 activity.

The above results on IAP interactions and their disruption during apoptosis were confirmed with immunoprecipitation studies. Similar to what was observed in the gel filtration studies, endogenous cIAP-1, cIAP-2 and XIAP co-precipitated with each other. Moreover, active caspase-3 was seen to co-precipitate with the IAPs upon apoptosis induction[1].

To conclude, the IAPs are present in large heteromeric functional complexes, where they work in a concerted manner to block the caspase activation. During chlamydial infection, cIAP-2 expression is increased, which strongly blocks caspase-3 processing. However, the presence of XIAP and cIAP-1, but not survivin, is important for cIAP-2 stability and apoptosis resistance in the infected cell.

How does the loss of Mcl-1 sensitize the infected cells to apoptosis, in spite of the up-regulation of cIAP-2? In the infected cells, increased amounts of cIAP-2 together with cIAP-1 and XIAP, strongly bind to active caspase-3. The block in the mitochondrial membrane permeabilization due to Mcl-1, leads to a reduced amount of Smac released into the cytosol upon apoptosis induction. This amount of Smac is evidently not enough to reverse the IAP-Caspase-3 interaction for apoptosis induction. However, in the absence of

[1] This work was performed by Rajalingam et. al. in the Rudel group.

Mcl-1, there is increased release of the IAP-antagonist Smac from the mitochondria leading to apoptosis induction by complete activation of caspase-3.

3.4 Modulation of the transcription factor HIF-1 to regulate survival genes

The transcription factor HIF-1 consists of two subunits- the constitutively active HIF-1β present in the nucleus and the hypoxia regulated HIF-1α present in the cytosol. During normoxic conditions, HIF-1α gets degraded, but upon hypoxia, it gets stabilized and translocates to the nucleus where it binds to HIF-1β to form the active HIF-1 . {Δερψ, 2005 3171 /ιδ}.

It was observed that HIF-1α was accumulated in the cytosol of infected cells during early time points of infection- around 12 hours. Quantitative Real Time PCR analysis revealed that the HIF-1α up-regulation did not occur at the mRNA level, suggesting that its accumulation is due to infection induced stabilization at the protein level. It is likely that the hypoxic conditions arising due to the onset of infection lead to HIF-1α stabilization. At later time points of infection, a broad scale degradation of HIF-1α was noticed. The initial accumulation and later degradation of HIF-1α correlates with previous reports on modulation of HIF-1α during *Chlamydia pneumonia* infection. Rupp et. al. showed that during early phase of *C.pneumoniae* infection there is stabilization of HIF-1α resulting in enhanced glucose uptake in the cells which would promote survival in the hypoxic conditions. However, during the late phase of infection, HIF-1α was targeted by the chlamydial protease-like activity factor (CPAF) secreted in the cytoplasm of the infected cells [124,124].

In the present work, immunofluorescence studies revealed a localization of HIF-1α in the nucleus of the infected cells. This event precedes the formation of HIF-1, which can consequently lead to increased expression of the anti-apoptotic Mcl-1 and cIAP-2 [112,116].

To check for the role of HIF-1α in apoptosis inhibition, RNAi mediated knock down was carried out. However, it was difficult to obtain a high level of knock down with the designed siRNAs. Consequently, only a slight increase in the apoptosis levels was observed in the absence of HIF-1α upon infection.

HIF-1 is a transcription factor which could lead to increased Mcl-1 and other survival genes expression, even when present in low amounts. Therefore, to analyze its role in apoptosis inhibition, a knock out cell line would be preferable.

Nevertheless, the effect of the knock down on the mRNA expression levels of Mcl-1 was measured with qRT-PCR. In a significant observation, depletion of HIF-1α abrogated the up-regulation of Mcl-1 mRNA upon infection.

To conclude, chlamydial infection leads to stabilization of HIF-1α and its translocation to the nucleus. It is possible that the infection leads to hypoxia in the cells, which would lead to the accumulation of HIF-1α. Silencing of *hif-1α* gene reduced the Mcl-1 expression which increased the rate of apoptosis in the infected cells, though by only a small amount owing to the low levels of knock down.

Previous reports have shown that severe hypoxia can promote apoptosis whereas mild hypoxic conditions (oxygen levels above 0.5 %) leads to its inhibition [118,119,120]. Dong et. al. have shown that cells under hypoxia undergo an induction of cIAP-2 [116]. Interestingly, they could see that the hypoxic cells were resistant to apoptosis induced by staurosporine, and this effect was dependent on cIAP-2 levels. It was also seen that the cytosol of the hypoxic cells were resistant to cytochrome *c* induced caspase-3 activation, supporting the evidence for an IAP induced block. Immunodepletion of cIAP-2 in the hypoxic cytosol reversed the block in caspase-3 activation upon cytochrome *c* treatment [122]. However, in the same study a mitochondrial membrane accumulation of Bax was reported to be suppressed on apoptosis induction. This block cannot be explained by the increased levels of cIAP-2 alone. It is likely that the inhibition of Bax accumulation was due to Mcl-1, which is also regulated by HIF-1α during hypoxia. Indeed, it has been shown elsewhere that HIF-1α dependent Mcl-1 expression is required for the anti-apoptotic effect of hypoxia [112].

These results confer accurately with the results showing that the HIF-1α dependant up-regulation of Mcl-1 and cIAP-2 can protect cells against apoptosis. Rupp et. al. have shown that *Chlamydia pneumoniae* can stabilize HIF-1α to regulate host cell glucose uptake during early infection [124]. The work from the present study shows that HIF-1α also has further, more crucial roles in the *Chlamydia* infected cells.

3.5 Different mechanism of apoptosis inhibition at later time points

TNF-α/CHX treatment at different time points of infection in Mcl-1 knock down cells showed that although the cells were sensitized to apoptosis after around 24 h post infection, they still resisted death after 48 h of infection. A closer look revealed that the size of the inclusion in the cell was crucial- cells carrying very large inclusions failed to get sensitized in Mcl-1 knock down cells, or after treatment with inhibitors. In fact, even the knock down of IAPs fails to sensitize such cells. This shows that there must be different mechanisms at play at later time points of infection, where the bacteria might directly interfere with the host apoptotic machinery.

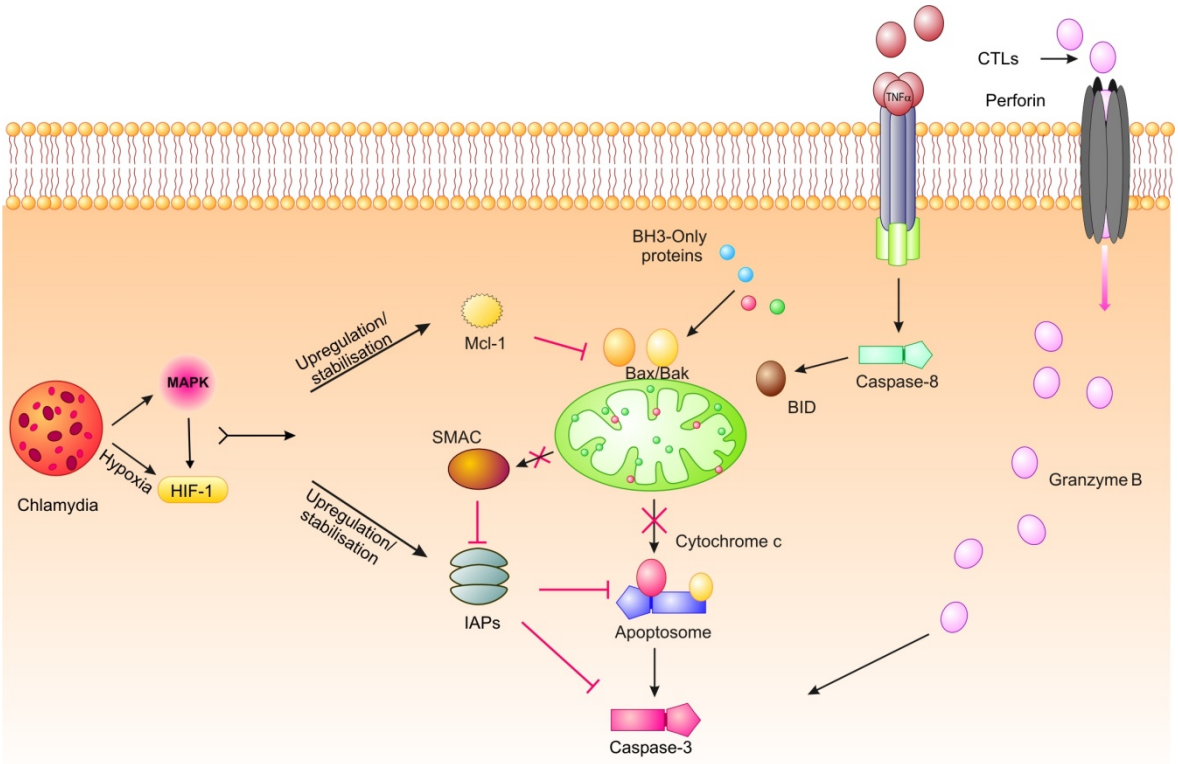


Fig. 37: *Chlamydia trachomatis* blocks apoptosis machinery of the host cell at different levels. During the early phase of infection, the transcription factor HIF-1 gets activated leading to the up-regulation of prominent anti-apoptotic proteins Mcl-1 and cIAP-2. It is observed that the over-espression of these proteins is dependant on the activation of MAPK pathways during the infection. Mcl-1 confers an active block in the apoptotic pathway at the mitochondrial level. Consequently, the release of cytochrome c and Smac from the mitochondria is blocked. The upregulation of cIAP-2 and the stabilization of the IAP–IAP complexes results in a direct block in the activation of caspase-3. The apoptotic pathway is thus obstructed at multiple levels leading to a strong protection

against apoptosis induced by different inducers including TNF-α/CHX, granzyme B or by intracellular stress.

3.6 Outlook

In the present study an attempt was made to understand the mechanisms of apoptosis resistance in the *Chlamydia* infected cells. Some key host cell factors responsible for the apoptosis resistance were identified. Further cell biology approaches revealed the signalling pathways activated upstream of these factors. Overall a more accurate picture was obtained, of infection mediated regulation of the apoptotic machinery inside a cell.

However, unsolved questions still remain in the bigger and more important picture of infection in the whole organism. What is the role of apoptosis resistance in the spread of *Chlamydial* infection in the host? Would the infection clear out faster if the cells in the system were sensitized to apoptosis, some way?

Work on knock-out mice could be one of the strategies employed. In the case of Mcl-1, where the knock out is lethal to mice, a localized knock down attained *in vivo* with specially delivered siRNAs could be a solution. This strategy is ideal since the cells from an infected tissue (e.g. in the lungs or the urinary tract) can specifically be targeted with siRNAs mediated knock down, while the other parts of the body are not harmed.

How effective is the resistance to apoptosis induced by cytoxic T lymphocytes (CTLs) in the spread of chlamydial infection in the body? It would be interesting to assess the infection in a Granzyme B/perforin knock-out mouse model, where the CTLs would be rendered ineffective of inducing apoptosis in the infected cells.

Tumor cells, especially in solid tumors, have a hypoxic environment and often show HIF-1α stabilization and subsequent HIF-1 transcriptional activity [176]. Further, a majority of human malignancies have an elevated expression of IAPs to promote tumor growth through the inhibition of cellular death [177]. Similarly, Mcl-1 has been shown to be up-regulated in numerous hematological and solid tumor malignancies [178]. Very similar conditions exist in the chlamydial infected cell, and it is tempting to hypothesize that the increased levels of the crucial anti apoptotic proteins during chlamydial infection would create conditions in the cell that help in the tumor maintenance and foster its growth. In fact there is growing evidence of the association of chlamydial infections with certain cancers, e.g. cervical cancer [179]. It is also probable that *Chlamydia* acts as a cofactor for

cancer causing agents, e.g. the Human Papilolma Virus, HPV. The knowledge of the factors responsible for the apoptosis resistance in the infected cells identified in this study would be crucial for research on *Chlamydia* induced tumor development.

4 Materials and Methods

4.1 Materials

4.1.1 Cell lines

Cell Lines	Sources
HeLa	Human cervical epithelial cells
HEp-2	Human larynx epidermal carcinoma cells
Jurkat cells	Human T cell Leukemia cell line
END1	Human endocervix; cervix

4.1.2 Cell culture media

Cell culture media including RPMI, MEM, DMEM were from (GIBCO BRL)

4.1.3 Chemical reagents

Chemical	Supplier
Agarose, low melting point	Sigma-Aldrich
Brefeldin A	Sigma-Aldrich
CCCP	Sigma-Aldrich
CD95 antibody	Immunotech
Cisplatin	Sigma-Aldrich
Cycloheximide	Sigma-Aldrich
ECL plus	NEN
Hoechst33258	Sigma-Aldrich
Mitotracker Orange	Molecular Probes
LY294002	Calbiochem
PFA	Roth
Protein A Sepharose	Amersham
Protein G Sepharose	Amersham
Staurosporin	Sigma-Aldrich
Thapsigargin	Alexis Biochemics
TNF-α	B D Pharmingen
U0126	Calbiochem
Mitotracker red	Molecular Probes
Hoechst 33342	Sigma-Aldrich

4.1.4 Primary antibodies

Antigen	Dilution for IF	Dilution for WB	Company
anti-chlamydial genus specific	1:100	-	Milan Analytica AG
Rabbit polyclonal, anti-human PARP		1:5000	SantaCruz
Mouse monoclonal, anti-human Hsp60	1:100	1:2500	Stressgen Biotech
Mouse monoclonal, anti-human α-tubulin	-	1:10,000	Cell Signalling

Mouse monoclonal, anti-human Cytochrome c	1:100	1:1000	Cell Signalling
Mouse monoclonal, anti-human cIAP-1	-	1:250	BD Pharmingen
Mouse monoclonal, anti-human cIAP-2	-	1:250	BD Pharmingen
Rabbit polyclonal, anti-human Cleaved Caspase-3	-	1:1000	Cell Signalling
Mouse monoclonal, anti-human SMAC	-	1:500	Cell Signalling
Mouse monoclonal, anti-human XIAP	-	1:250	BD Pharmingen
Mouse monoclonal, anti-human MCL1	-	1:1000	BD Pharmingen
Mouse monoclonal, anti-human HIF-1α	1:100	1:1000	Alexa
Mouse monoclonal, anti-human Lamin	-	1:2000	SantaCruz
Mouse monoclonal, anti-human Caspase-9	-	1:1000	Upstate

IF-immunofluorescence, WB- western blots

4.1.5 Seconday antibodies

ECLTM sheep anti-mouse peroxidase	1:3000	Amersham Bioscience
ECLTM donkey anti-rabbit peroxidase	1:3000	Amersham Bioscience

4.2 Buffers

4.2.1 Cell culture media for *Chlamydia* culture and maintenance

Growth medium	MEM, 1 % non essential amino acids, 2 mM L-Glutamine, 10 % FCS, 10 mM HEPES and 10 µg/ml gentamycin.
Maintenance medium	MEM, 1 % non essential amino acids, 2 mM L-Glutamine, 10 mM HEPES, 10 µg/ml gentamycin and 1 µg/ml Cycloheximide.
Infection medium	MEM, 1 % non essential amino acids, 2 mM L-Glutamine, 10 mM HEPES and 5 % FCS

4.2.2 Indirect Immunofluorescence

4 % PFA, pH 7.4	Dissolve 4 g of PFA in 50 ml H_2O. Heat to 60°C while stirring and add 1 ml 1 M NaOH. Add 10 ml 10 x PBS, cool down to RT and adjust pH to 7.4 (ca. 1 ml 1 M HCl) Make up the final volume to 100 ml and freeze in aliquots at -20°C
10x TBS	200 mM Tris pH 7.5, 1.54 M NaCl, 20 mM EGTA, 20 mM $MgCl_2$
Moviol	13.3 % (w/v) Moviol 4-88, 33 % (w/v) Glycerin in 0.15 M Tris-HCl, pH 8.5

4.2.3 Isolation of mitochondria

Solution A	Solution B containing 2 mg/ml BSA
Solution B	20 mM Hepes-KOH pH 7.6, 220 mM Mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF
Sucrose Buffer	10 mM Hepes-KOH pH 7.6, 0.5 mM sucrose

4.2.4 SDS-PAGE and Western blot

2x sample buffer	20 % Glycerin, 100 mM Tris-HCl pH 6.8, 4 % SDS, 200 mM DTT, 0.2 % bromophenol blue
Running buffer	0.25 M Tris, 1.918 M Glycin, 1 % SDS
Transfer buffer	50 mM Tris-HCl, 40 mM Glycin, 20 % Methanol

TBS-T	10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 % Tween-20
Blocking solution	10 % milk powder in 3 % (w/v) BSA in TBS-
Stripping buffer	62.5 mM Tris-HCl, pH 6.7, 10 mM β-Mercaptoethanol, 2 % (w/v) SDS, 0.05 % Tween-20

4.2.5 Protein staining / fixation

Ponceau S	2 % PonceauS (w/v), 30 % TCA (w/v), 30 % sulfosalicylic acid (w/v)
Coomassie solution	0.25 % (w/v) Coomassie Brilliant Blue R250, 50 % Methanol, 10 % glacial acetic acid
Destainer	10 % ethanol, 10 % glacial acetic acid
Fix-solution	25 % methanol, 10 % glacial acetic acid

4.2.6 Immunoprecipitation

Chaps buffer	150 mM NaCl, 10 mM HEPES pH 7.4, 1 % Chaps (w/v), protease inhibitors
Lysis buffer	0.4 % Triton X-100, 20 mM Tris pH 7.2, 2 mM EDTA pH 7.2
SPG buffer	75 g /l sucrose, 0.52 g/l KH_2PO_4, 1.22 g/l Na_2HPO_4, 0.72 g/l glutamic acid, 10 % FCS
RIPA buffer	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 % NP-40, 0.5 % Triton X-100, 1 mM $NaVO_3$, 10 mM Na-pyrophosphate, 1 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, Protease inhibitor mix
Protease inhibitor	1 g/ml of Aprotinin, 0.5 g/ml Leupeptin, 1 mM Pefabloc, 10 M Pepstatin
MB buffer	400 mM Sucrose, 50 mM Tris, 1 mM EGTA, 5 mM β–mercaptoethanol, 10 mM KH2PO4, pH 7.6, 0.2 % BSA
NB buffer	10 mM HEPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM Pefablock

4.3 Methods

4.3.1 Growth and purification of *Chlamydia*

Before harvest, bacteria were propagated in HeLa cells, grown in six well plates with Maintenance medium, MM, by incubating them at 37°C for approximately two days. In case of *C.pneumoniae* infection the plates were centrifuged at 100 X g for 1 h to enhance the infection. After 55 to 70 hour post infection, MM was poured off and the cells were detached in cold HBSS (Biochrom) using 3 mm diameter glass beads or rubber police man. The cell suspensions were centrifuged at 500 x g and 4°C for 10 min (Hermle Labortechnik, Wehingen, Germany) and the pelleted cells were then combined together and ruptured using glass beads and a Dounce homogeniser to ensure the breakage of cells. The lysates were centrifuged as before to sediment nuclei and cell debris. The supernatant was removed and recentrifuged at 20,000 x g for 40 min at 4°C in a SS-34 rotor (Sorvall Instruments, CT, USA) to pellet chlamydiae. The crude bacterial preparations were suspended in 30% Percoll (Vol/ Vol) (Pharmacia, Uppsala, Sweden) in 10 mM HEPES, 145 mM NaCl, pH 7.4 by repeated passages in 22 gauge needle and centrifuged again at 90,720 × g (TFT65.13 Rotor, Kontron Instruments, Zürich, Switzerland) for 40 min at 4°C. The harvested bacteria were washed once in sucrose phosphate glutamate buffer (SPG pH 7.4), diluted and resuspended in the same buffer.

Chlamydia suspensions were then aliquoted and frozen at -75°C and freshly thawed before each experiment.

Inclusion count

The inclusion forming units per ml has been calculated as follows. The EB suspensions were diluted 5 or 10 fold and added onto the host cell monolayer and the plates were centrifuged at 100 X g for 1 h to enhance the infection. After adding approximately 1 ml of infection medium (IM) to these wells, plates were incubated for 48 h at 37°C and 5% CO_2. After incubation, the cells were permeabilised and stained with the chlamydial genus specific antibody (Table1). The stained inclusions were counted at 400 X magnifications and the area of the microscopic field used with a micrometer slide (A) was estimated. The

total area of the monolayer was estimated as well (B). Fields in the monolayer C= B/A. The number of IFU/ ml can be calculated as follows:

$$\frac{\text{No of IFU counted}}{\text{No of Fields examined}} \times C \times DF \times V$$

DF- dilution factor of EB preparation

V- factor determined by the volume of the inoculum

4.3.2 Indirect immunofluorescence

The cells were washed with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature. The fixed cells were washed with PBS twice for 5 min either by placing the plates on the moving platform at a minimal speed or by rinsing them by tilting the plates. The following solutions were prepared:

Solution A: 0.5 % of Triton X-100 in PBS,

Solution B: 0.05 % of Triton X-100 with 0.2 % BSA in PBS and

Solution C : 1 % Goat serum with 0.05 % of TritonX-100 in PBS.

The cells were then permeabilised with Solution A for 5 to 10 min and washed with PBS twice followed by incubation with Solution B for 15 min at room temperature. It was then washed away by rinsing with PBS twice. The cells were further incubated with solution C for 20 min at room temperature. After washing with PBS once the primary antibody at the recommended dilution was prepared in solution C. About 25-30 µl of the primary antibody mix was then carefully added onto the cover slips and incubated at room temperature for one hour under moist conditions. After incubation, the cells were washed with PBS thrice and in the meantime the secondary antibody mix in solution C was prepared and added onto the cover slips after drawing all the PBS out. After incubating for 40 to 50 min the cells were washed and mounted onto glass slides with Moviol. In case of double staining the same procedure as mentioned before was repeated with another set of primary and secondary antibodies. For visualization of the mitochondria Mitotracker Red was added to living cells prior to fixation for 20 min at 37°C.

4.3.3 Smac release assay

This method is based on the fact that Smac has a mitochondrial localization in untreated cells which results in a punctuate staining. After mitochondrial outer membrane

permeabilisation (MOMP), Smac localises to the cytosol resulting in the loss of the punctuate staining. This can be monitored microscopically.

Control and Mcl-1 knockdown cells were grown on coverslips and infected with *C. trachomatis*. 24 hours post infection, apoptosis was induced in infected and control cells with TNFα and cycloheximide. After six hours, the cells were fixed with 4 % PFA/PBS. The cells were then washed once with PBS and permeabilised with 1 % Triton/PBS for 10 minutes. Blocking was done using 1 % BSA and 0.05 % Tween 20 in PBS, for 30 minutes. The samples were incubated overnight with a mouse monoclonal anti-human Smac antibody (BD Pharmingen) at a dilution of 1:100 in PBS. The samples were washed twice with PBS and the bound antibodies were detected using an anti-mouse Cy-2 conjugated secondary antibody.

The coverslips were examined with a Leica confocal microscope using the TCS software in accordance with established methods. Five fields were selected randomly for each sample and cells which have lost the punctuate staining were digitally recorded and the percentage of cells which have lost Smac staining in relation to those who who retained it were calculated.

4.3.4 Induction of apoptosis

To induce apoptosis, either 1 μM staurosporine (Sigma) or 40 ng/ml of TNFα (Pharmingen, San Diego, California) together with 2 μg/ml of cycloheximide were applied to the cells for four to six hours. The control cells were incubated with cycloheximide alone.

4.3.5 Transfection of siRNAs and subsequent infection

To inhibit expression of genes by siRNAs, 50,000 cells/well were seeded in a 12-well plate at least 20 h prior to transfection. Short interfering RNAs designed for the inhibition of the genes under investigation and for luciferase (siLuc) as negative control were transfected using the Transmessenger transfection kit or the RNAiFect transfection kit (Qiagen). One day post transfection, the nearly confluent cells were infected with *C. trachomatis* and 24 h later the samples were analysed by immunoblot and apoptosis analysis. SiRNAs targeting the following sequences were employed in this study:

siLuc- 5'-AACUUACGCUGAGUACUUCGA-3',

siMcl-1 [2] 5'-AAGAAACGCGGUAAUCGGACU-3',
siMcl-1 [3] 5'-AAGGACACACAAAGCCAATGG-3',
siBim 5'-CGGAGACGAGTTTAACGCTTA-3',
siBad 5'-ACGAGTTTGTGGACTCCTTTA-3',
siPuma 5'- CAGCCTGTAAGATACTGTATA-3',
siBid 5'-TAGGGACTATCTATCTTAATA-3'.
For silencing of Mcl-1, both siRNAs were transfected together at a final concentration of 60 nM each.

4.3.6 Generation of cell lines expressing Mcl-1 shRNAs

Stably shMcl-1 expressing HeLa cells were generated according to http://www.tronolab.com. 293T cells were transfected by calcium phosphate method with the target vectors pLVTH-M-shMcl and viral packaging vectors. After 48 h, lentiviral supernatants were filtered through a 0.45 μm filter (concentrated by ultracentrifugation at 25,000 rpm) and infections of HeLa cells were performed in the presence of polybrene (Sigma). 8 h post infection medium was exchanged and after 48 h GFP-positive cells were enriched by FACS sorting (FACSDiva, Becton Dickinson). Single clones of GFP-positive cells were lysed in sample buffer and the efficiency of gene silencing was checked by quantitative realtime PCR and immunoblot analysis as mentioned above. As control, HeLa cells were transduced with the empty vector and all cell lines were tested for interferon response to exclude non-specific effects. The sequence targeted by the mature shMcl RNA in the Mcl-1 mRNA was: 5'-UAGAGUGUAUACAGAACGAAU-3'.

4.3.7 Apoptosis assays and quantification

Detection of chromatin condensation

Cells were seeded in 12 well plates on cover slips infected, and treated with apoptotic inducers as described above. After the specific time points the cells were washed twice with 1 X PBS and fixed at room temperature with 4 % PFA for 30 min. After twice washing with PBS, the cells were stained with Hoechst 33342 (1 mg/ml) for 15 min in dark. After two washes with PBS the cover slips were dried and mounted onto glass slides using Moviol and viewed with an Axiovert Fluorescence microscope at 450- 490 nm.

Different fields were then photographed through the connected digital camera. For quantification cells from five random fields for each sample were counted using a 40X objective lens. The number of apoptotic, nonapoptotic and the infected cells were counted and the percentage of apoptotic cells was calculated.

Detection of caspase-3, cytochrome c and TUNEL assay

Following the induction of apoptosis, the infected and uninfected cells were first subjected to the detection of *C. trachomatis* using specific antibodies. After being fixed in 4 % PFA, and washed with PBS, cells were incubated for 1 h with the rabbit polyclonal antibody specific for activated caspase-3, used at a final dilution of 1:100 (a gift from A. Srinivasan, Idun Pharmaceuticals, San Diego, California.). This was followed by incubation for 1 h with goat anti-rabbit immunoglobulin G coupled with DTAF (Dianova). The Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling (TUNEL) reaction was performed using the Apoptosis Detection System, Fluorescein, strictly in accordance with the manufacturer's instructions (Promega, Madison). Cytochrome c was detected with the mouse monoclonal antibody against holocytochrome c (Pharmingen).

4.3.8 Preparation of cell lysates for immunoblotting

Cells were trypsinised and washed twice with cold PBS at 4°C. Cell pellets containing 2×10^5 cells were resuspended in 50 µl lysis buffer (62 mM Tris-HCl, 2 % SDS, 10 % Glycerol, 5 % β–mercaptoethanol pH 6.8) or in single detergent buffer (20 mM Tris- HCl pH 7.5, 150 mM NaCl, 1 % NP-40, 1 µg/ml Aprotinin, 0,5 µg/ml Leupeptin, 1 mM Pefabloc, 10 µM Pepstatin) and heated in a boiling water bath or a heating block for 10 min. Cell lysates were removed by centrifugation (13000 rpm at 4° C) and the lysates were stored at – 20°C for further analysis.

4.3.9 SDS-PAGE and immunoblotting

Proteins were separated under reducing conditions for 1 h at 120 V. The gels were blotted overnight at 100 mA onto PVDF membranes (Millipore). Prior to this the PVDF membranes were activated by incubating them with methanol for a minute and then washed with TBST for 5-10 min. Membranes were blocked for 2 h in 3 % BSA in Tris-buffered saline (TBS)/0.1 % Tween 20 (TBST) and then incubated for 1-2 h with suitable first antibody. After washing the blots three times for 10 min in TBST they were incubated

for another hour with the peroxidase coupled secondary antibody and the bound antibody was detected by enhanced chemiluminescence (NEN). The chemiluminescence was detected by exposing to a BioMac™ MR film (Eastman Kodak.Co.). The blots were sometimes stripped and probed with other antibodies. For stripping, the PVDF membranes were again activated by incubating them with methanol for a minute and then washed with TBST for 5-10 min. Then about 200 ml of stripping solution (62,5 mM of Tris-HCl pH 6,7 with 10 mM β–Mercaptoethanol and 1 % SDS in distilled water) was added to these blots and incubated in a shaking waterbath at 50°C for 30 min. The blots were washed well with TBST for 1-2 h by changing TBST for every 20 min. The stripped blots were then incubated with 3 % BSA in TBST and the same procedure was followed as mentioned above to reprobe them with a new antibody.

4.3.10 Quantification of immunoblot data

The blots were scanned using a Canon Scan 5000F scanner. Quantification was done by densitometry analysis using Adobe Photoshop CS3 (Adobe Systems Incorporated). An area around each band was selected and the mean intensity was calculated by the software. The mean intensity of a region on the blot near the bands was taken as a "blank". The total intensity of any band was then calculated as the difference between the mean intensity of the band and the blank, multiplied by the pixel size of the area selected around the band.
The total intensities of bands were normalized using the Actin control. The values were plotted on a relative scale with a value of 100 % assigned to the 0H value in each graph.
Preparation of mitochondria free cytosolic fractions from cell extracts
Hela cells were cultured in 150 cm^2 flasks and harvested. The cells were pelleted by centrifugation for 5 min at 1200 RPM and 4 ºC. The pellet was washed with PBS and then resuspended in MB buffer (400mM Sucrose, 50mM Tris, 1mM EGTA, 5mM ß-mercaptoethanol, 10mM KH_2PO_4, pH 7.6). The suspension was kept on ice for 20 minutes and the cells were then disrupted by pottering.
The nuclei and cell debri were expelled by centrifugation at 3000 RPM for 2 minutes. The supernatant was centrifuged again at 10,000 RPM for 10 minutes to remove the mitochondria.

4.3.11 Gel filtration chromatography

The mitochondria free cell cytosolic fraction isolated above, was applied to a Superdex 200 HR 26/60 column (Amersham Pharmacia) at a flow rate of 1 ml/min. The column was equilibrated with PBS buffer. Fractions, each 5 ml in volume, were collected starting at 80 ml. The column void volume was 100 ml.

The fractions were mixed with an equal volume of 25 % trichloroacetic acid and kept on ice for 30 minutes. These were then centrifuged at 4000 RPM for 30 minutes. The supernatant was carefully discarded and the pellet washed twice with acetone. The pellet was then dissolved in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting.

4.3.12 Softwares used

Windows 2000, XP (Microsoft)
MS Office (Microsoft)
Mozilla Firefox
Adobe Acrobat 7.0 (Adobe)
Photoshop 6.0 (Adobe)
Corel Draw 11 (Corel)
ACT-1.2 (Nikon)
Visicapture (Scion Corp.)
Reference Manager 11 (Thompson ISI research soft),
CellQuest Pro (BD)
Image Reader LAS-3000 (Fujifilm Life Science)
BasReader 3.14 (Fujifilm)
Aida Image analyzer 4.03 (Fujifilm)
Vector NTI 10 (Invitrogen)
Chromas (Technelysium Pty)
QIAsoft 4.1.4.7 (Qiagen), SDS 2.2.2 (Applied Biosystems), ImageJ

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